A Study of Antibiotic Compounds in New Zealand Plants

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"Inspite of much fumbling,
Science has given us priceless
remedies, many of them derived
from the plant lore of people
quite innocent of Science"

- Norman Taylor 1965

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ABSTRACT

The antifungal polyacetylene, falcarindiol, ((9Z) - heptadeca -1,9-diene-4,6-diyn-3,8-diol) was isolated from the New Zealand native plant *Schefflera digitata* (Araliaceae). The structure was established by CIMS, ^{13}C-NMR and ^{1}H-NMR. Falcarindiol was found to be present in the leaves at a concentration of 0.15 to 0.17% of the fresh weight. This compound exhibited selective antifungal activity against dermatophytes with minimum inhibitory concentrations of 5 µg/ml against *Microsporum canis, M. gypseum, M. nanum, Trichophyton mentagrophytes, T. rubrum, T. erinacei, T. tonsurans* and *Epidermophyton floccosum,* and causes gross disorganization of sensitive fungal spores. The spectrum of antibiotic activity of falcarindiol against other micro-organisms and also the screening of New Zealand *Pseudopanax* species for antifungal activity are reported.

1.0 INTRODUCTION

As a result of a preliminary screening programme carried out in the Botany Department of the University of Canterbury, interesting biological activity has been found in a number of New Zealand native plants. One of these plants, *Schefflera digitata*, was selected for further study with the aim of identifying the active compounds, because initial experiments (41, 114) had indicated pronounced antifungal activity.

Although there are a small number of effective antifungal agents currently available, there are still a number of dermatophytic infections, particularly those caused by *Trichophyton rubrum* which often prove difficult to treat. Moreover the treatment of deep seated and systemic fungal infections is still unsatisfactory with very few treatment options available.

With this background it was decided to investigate further this pronounced antifungal activity of *Schefflera digitata*, activity which first came to light as a result of examining records of Maori folk medicine.

1.1 Plants and Folk Medicine

The development of some form of folk medicine is probably an integral part of the development of any group or tribe of people. It is a biological axiom that all living organisms, man included, are subject to various biological controls which tend to eliminate the weak and thus exercise some control over the absolute numbers of individuals.

It is therefore not surprising that a feature of man's development is that he has sought to try to modify many of these controls by cultivating plants and domesticating animals. In more recent times man has attempted, with a considerable measure of success, to reduce the impact of fungal and bacterial diseases on the survival rate of his species.

Prior to the discovery of bacteria and the development of modern concepts of disease mankind had to rely on less reliable ideas and concepts of what caused diseases and how to treat them. The developments of these concepts relating to disease and death played an important part in the success or otherwise of the cure's developed. Some tribes developed ideas which recognised that physical and environmental factors were possibly involved and so encouraged

experimentation. Other tribes developed concepts which placed the cause of disease in the spiritual world, so that appeasement of the spirit was attempted rather than treatment of the disease itself; a consequence of this philosophy was that experimentation tended to be discouraged.

These two concepts represent the extremes and there are many groups which lie in between them. In many parts of the world native cultures, such as those of West Africa, Central and South America and India, developed a folk medicine based on experimental as well as spiritual concepts with the result that a practical and effective folk medicine developed. This is in marked contrast to the indigenous Maori people of New Zealand who developed strong spiritual concepts surrounding sickness and death, equating illness with infringement of "tapu" or the result of a curse. These concepts appear to have inhibited the development of a functional folk medicine until the advent of Europeans. (see 1.2)

The Maori also failed to appreciate the practical possibilities of using poisonous plants to kill fish, birds and animals, and this may be due in part to the lack of grazing mammals and large fresh water fish, other than eels, in New Zealand prior to the arrival of Europeans.

Another important characteristic of folk medicine is the type of afflictions which are treated "successfully" (105). In general these are physical disorders where the state of the patient's mind may be as important as the condition from which he suffers. For example, in many chronic or incurable conditions and many psychosomatic conditions, the affliction will either heal itself or come and go irrespective of the medicine used.

Despite the apparent lack of development of a practically based folk medicine by the pre-European Maori, a large number of 'folk cures' have been recorded by Brooker and Cooper (43). One plant, *Schefflera digitata* ("seven finger" or "Pate"), is reported to have been used by the Maori to treat ringworm infections.

1.2 Maori Medicine

There seems to be considerable confusion regarding the status of the folk medicine of the pre-European Maori. The author of a popular book,Christina Macdonald (120), seems totally convinced that herbal remedies were an important part of Maori folk medicine. "It would be difficult to find anywhere in the world a race of people, other than the Maori, who made such intelligent use of primitive materials" she writes, yet the Maori made no use of tutu, and uncooked Karaka kernels although they were well aware of their poisonous nature. Elsdon Best (18), having taken a closer look at Maori culture generally, was of the opinion that herbal medicine is very definitely a post European development. According to Best (18, 19) and Buck (44) the Maori's spiritual concept of disease and death was such as to discourage the development of herbal medicine.

To quote Elsdon Best, "The Maori firmly believed in demonical possession. He not only personified disease and sickness generally, and so spoke of being afflicted by Whiro, Maiki-nui and Maiki-roa (Whiro - (Darkness) was the origin of all diseases), but also believed that, when ill, some atua had taken possession of his body." (18) This demonical possession may be caused by either black magic or violation of some law of tapu by the sufferer. The tohunga who would have been consulted when illness occurred would first determine the cause of the patient's illness, i.e. what wrong act he had committed and for which the gods were punishing him. Having established which god was offended he would then perform the necessary ritual to exorcise the demon; any treatment as such was purely empirical.

The above is in marked conflict with many of the observations of Cook, (56) Cruise (60) and Colenso (54) made in earlier times. The difficulties may lie in the sources of the information. Best's writings are probably based largely on information supplied by tribal elders and Tohunga's while other observations may have been of the healing practices and philosophies of Maori women. This conflict may never be resolved as Maori culture and custom have been modified considerably since contact with Europeans began.

There are however two important features of Maori folk medicine as we now know it (43, 120). Firstly, Maori folk medicine was more efficient at treating minor and superficial ailments such as warts, boils and open wounds, than internal disorders and major ailments.

The only successful treatments for the latter diseases were for constipation and diarrhoea (43, 44). Heat treatments and medicinal steam baths were often prescribed for gynacological and muscular problems. (43) The singular lack of success of post European tohunga's and other practitioners of Maori Medicine led to the Tohunga Suppression Act of 1907 (43) designed to reduce the impact of what was by then a large scale fraud.

The second aspect is illustrated by the confusion surrounding the records of Maori folk medicine. Brooker and Cooper (43) have collated and documented virtually all that is known about Maori Medicine and a noticeable feature is that many plants are prescribed to treat the same condition and the same plant may be prescribed for a great diversity of ailments! This does not inspire confidence especially as most of the plants are reasonably widespread in New Zealand and would have been readily available to most Maori tribes in pre-European days. This does not mean that the remedies mentioned have no worth or basis, but rather it indicates that Maori folk lore medicine (compared to the folk medicine of some other ethnic groups) is probably not a reliable guide to start a programme to screen plants for pharmaceutical and chemotherapeutic agents.

1.3 Plants as a source of Pharmaceuticals

Man's first practical pharmaceuticals were largely derived from herbs and other plants. Early tribal groups, particularly those which developed a written language (e.g. the early civilisations of Greece, Egypt and China), but also the early civilisations of Central and South America and West Africa, developed a practical knowledge of the useful properties of many plants. The Greeks in the third century B.C. knew how to collect, prepare and use opium, the Inca's knew of the properties of *Cinchona* bark (quinine). The ancient Hindu's used *Rauwolfia* to treat conditions similar to those which are treated today by the *Rauwolfia* alkaloids. The South American Indians chewed Coca leaves and today the pure compound, cocaine, is used.

Many of these plants were investigated by the early analytical chemists, who isolated such useful compounds as Cocaine (1860 - from *Erythroxylon Coca*), reserpine (*Rauwolfia*) Quinine (*Cinchona*) digitalis (*Digitalis purpurea*) Curare, salicylic acid (*Salix* sp) and Hydnocarpus oil. (112) The history of the use of many of these plants and the isolation of their active principles has been reviewed

by Taylor (169) and Kreig (110), whilst Lewis and Elvin Lewis (115) have produced a comprehensive review of medicinal plants.

Plants figured large in both research and the use of pharmaceuticals until the middle of the 20th century (112). Following the discovery of the antibiotic Penicillin, much of the emphasis switched to micro-organisms as a source of antibiotics since they were generally easier to grow in large quantities and it was much easier to recover the biologically active compound from them than from plant material. This switch in emphasis is still reflected in the division of money spent on pharmaceutical research (70). Despite this disproportion in the allocation of research funds, medicinals derived from plants are still widely used and are staging a comeback due to the increased use of steroids (e.g. in oral contraceptives) and alkaloids.

In a recent review, Farnsworth (70) commented that in 1973 25% of all prescriptions issued in the U.S.A. contained preparations from higher plants (see Table 1.1) and in addition, vast quantities of non-prescription preparations derived from plants are sold each year.

Table 1.1

The most commonly encountered pure compounds derived from higher plants used as drugs in 1973 in the U.S.A.

Active Principle	% of total prescriptions
Steroids (95% from diosgenin)	14.69
Codeine	2.03
Atropine	1.50
Reserpine	1.45
Pseudoephedrine*	0.90
Ephedrine*	0.77
Hyoscyamine	0.75
Digoxin	0.73

* Produced commercially by synthesis

Source: Farnsworth & Bingel (70)

There are an estimated 250,000 - 500,000 plant species on this planet of which less than 20% have been examined for pharmaceutical and chemotherapeutic compounds (less than 5% thoroughly) (Table 1.2) so the scope for further investigation is limited only by time and money.

The true situation regarding the search for medicinals from plants is probably unobtainable because it is well known that secrecy and/or protection of information is necessary if commercial development is to be possible. Furthermore since patenting of the isolation of naturally occurring compounds is difficult, if not impossible, it is very likely that the biological activity of a number of natural products is being kept secret until such time as it is possible to synthesis the compound (a process which is patentable). Thus all one can do is comment on the published literature and acknowledge that much more may be known about antibiotics and chemotherapeutic agents from plants than is public knowledge.

Table 1.2

Types of Biological Activity possessed by Plant Products

A Pharmacological	Citations; 1975 literature
Anti-inflammatory Agents	32
Anti-ulcer agents	12
CNS active agents	14
Hypocholesterolemics	22
Toxic plant principles	12
Cytotoxic agents	49
Tumor promoters	25
Others	104
B Chemotherapeutic	
Antibacterial	30
Antifungal	28
Antiprotozoan	30
Tumor inhibitors	63
Others	15

Source: Fransworth & Bingel (70) Table 8

New Zealand's unique flora has been the subject of a number of chemical investigations and Cambie and his co-workers have recently published a valuable phytochemical register of New Zealand plants (47).

Investigations of the New Zealand flora for biologically active compounds have received even less attention than those of other countries despite the high proportion of endemic plant species. These investigations that have been carried out were documented by Fastier and Laws (73) in 1975. Since then no further work on the biological activity of the New Zealand flora has been published.

1.4 Screening of Plants for Antifungal Activity

Screening plants for antifungal activity requires that the plant extracts be tested against a variety of different test organisms, because the fungi which are pathogenic to plants, animals and humans are a diverse group of organisms. The problems associated with antifungal screening are further compounded by the different forms of inoculum possible, a result of distinct phases in the life cycle of the fungi (which are absent in bacteria) namely: the resting spore (often with a thickened cell wall), thin walled vegetative spores produced under favourable conditions, and the mycelial phase itself.

In the case of human fungal pathogens there is an added constraint because both the fungal pathogen and the human host are eucaryotes while bacteria are procaryotic. Thus there is a greater similarity between host and pathogen than is the case between bacteria and their human hosts, this means that many promising antifungal agents also have potentially fatal consequences for the host.

The search for antifungal agents from plants can be divided into screening for two distinct types of activity. Much work has been concentrated on the search for phytoalexins and natural products with antifungal activity against phytopathogens (7, 13, 68, 74, 79, 81, 97, 103, 104, 125, 163, 170, 175). In this area a number of interesting compounds with marked activity have been found although none of them have yet been developed as commercial fungicides. Those compounds which are produced in response to pathogenic attack (phytoalexins) are of considerable interest to the plant breeder who can try and select for the ability to produce the phytoalexin.

The other major area of antifungal research is aimed at finding compounds which will inhibit the growth of human fungal pathogens (see 1.5) (77, 84, 85, 97, 171). The division between plant fungal pathogens and human fungal pathogens is not always distinct with some fungi (e.g. *Aspergillus* spp) attacking both plants and humans. Thus although the fungal pathogen may be the same, the respective hosts are distinctly different and an antifungal agent useful for one is often totally unsuitable for the other.

There are also specific nutritional requirements and cultural conditions for different groups of fungi. For example the dermatophytic fungi have specific nutritional requirements, whilst saprophytic fungi have less precise nutritional needs. There are, therefore sufficient differences between phytopathogenic fungi and human pathogenic fungi to consider them separately.

The search for compounds with activity against human fungal pathogens has been the predominant theme of this project. Much of the early work in this area involved screening large numbers of plants and plant extracts against a relatively limited range of test organisms. Large numbers of plants have been screened using this approach (20, 21, 71, 72, 78, 83, 99, 130) and activity found in a number of species, but the follow up of this work has been disappointing with few of the plants exhibiting activity having been studied in depth. Since it is not usually possible to test for activity against a wide range of different micro-organisms in this type of system, any activity discovered is likely to be of a very general nature and not the more selective type of activity which is now sought in new antibiotics.

Another approach to this problem, that of phytochemical screening, is based on pre-existing concepts of the types of molecules possessing biological activity. It involves the search for compounds chemically related to a compound known to possess biological activity. The main chemical groups sought are the alkaloids (127, 185), flavanoids (175) and saponins. Farnsworth (69) has produced an excellent review of biological and phytochemical screening of plants, including a detailed evaluation of the screening tests used. This approach has produced a number of useful compounds (mainly with pharmacological activity as opposed to chemotherapeutic activity) but has the obvious disadvantage of missing completely any novel biologically active compound.

The third approach has been to invoke some form of pre-selection of the plants prior to a more detailed examination of a limited number of species. In the search for antifungal compounds to combat human pathogens, folk lore medicine is an obvious form of pre-selection. Examples of this approach include the work of Fuijta *et al* (77), Disalvo (63), Acharya and Chatterjee (1), Das *et al* (62), Ieven *et al* (94), Lalithakumari *et al* (113) and Swartz and Medrek (165).

Other workers screening plants for antifungal agents, have examined plants with known biological activity (135, 173), while others have followed up the positive indications of antifungal activity discovered in preliminary screening tests (84, 97, 88, 95, 96, 103, 143, 171).

Recent reviews of the antimicrobial and antifungal compounds isolated from plants include those by Sehgal in 1961 (158), Stoessel in 1970 (162) and Mitscher in 1975 (126), dealing mainly with the chemistry of the active principles.

1.5 Human Fungal Pathogens

When compared to the human mortality caused by bacteria, viruses and protozoa, mortality due to fungal diseases is almost insignificant. The incidence of fatal fungal diseases is fortunately small but the nuisance value of superficial fungal infections is none the less considerable. Superficial fungal infections can have a considerable effect on the ability of people to work and can be a major problem for the armed forces. In the Vietnam War for example, superficial dermatophyte infections were the largest single cause of 'non-combat' man-days lost by the U.S. Army and thus was a major health problem. (26)

The incidence of deep-seated fungal infections such as histoplasmosis, blastomycosis and coccidioidomycosis caused by the fungi *Histoplasma capsulatum, Blastomyces dermatitidis, B. brasiliensis* and *Coccidioides immitis,* is rare except in some tropical areas particularly southern U.S.A., Central and South America. This is fortunate, for there is no effective treatment for many of these conditions (3, 82, 128, 146).

The second group of fungal infections are due mainly to opportunistic colonisation of human tissue by yeasts and saprophytic filamentous fungi other than the dermatophytes (51). This group includes such organisms as *Candida albicans, Aspergillus* and *Mucor* spp.

C. albicans causes various forms of candidosis including 'thrush', and often proliferates rapidly in the upper respiratory and intestinal tracts. Nowadays this appears to be an almost inevitable complication of broad spectrum antibiotic therapy, and *C. albicans* infections may also occur in the body cavity as a result of surgery on the intestinal tract since it is normally present in the intestinal microflora. *Aspergillus* species occasionally establish themselves as pathogens as a complication of pre-existing diseases such as bronchietasis, tuberculosis and cystic fibrosis. There is a close similarity of the symptoms of *Candida* and *Aspergillus* infections to those of the dermatophytes with the result that diagnosis of the causal agent is sometimes incorrect and subsequent treatment of little value as there is no single form of treatment for all three types of fungal diseases.

The third group of human fungal pathogens are the superficial skin fungi known as the dermatophytes (1.5.1). Whilst it is rare for these fungi to cause fatal infections, they are sometimes difficult to control and in some cases it is impossible to effect a cure.

Dermatophytosis is the strict term for superficial skin infections caused by the dermatophytic fungi, (i.e. fungi capable of utilising keratin) while the more general term Dermatitis includes skin inflammations due to allergic reactions to various chemical agents, photodermatitis, and many stress related skin inflammations. However, many of these inflamed areas may subsequently be invaded by opportunistic fungi.

1.5.1 The Dermatophytic fungi

1.5.11 Classification

The dermatophytic fungi are characterised as a group of fungi capable of infecting keratinized areas of the body and utilising keratin. These fungi are grouped into three genera within the Fungi Imperfecti: *Microsporum* (14 recognised species) *Trichophyton* (20 recognised species) and *Epidermophyton* (1 species) (2) (form class Deuteromyceses - form family Moniliaceae).

The corresponding perfect states known are *Arthroderma* (Trichophyton) and *Nannizzia* (Microsporum) (2). This means that the dermatophytes may be classified in the family Gymnoascaceae, order Eurotiales, sub class Euascomycetidae, within the Class Ascomycetes (4).

The dermatophytes can also be subdivided on the basis of their epidemiology. Ajello (3) divides the dermatophytes into three groups according to their host preference.

(1) *Anthropophilic* - parasites of man not known to exist as saprophytes in soil and seldom found to infect lower animals.

(2) *Zoophilic* - basically parasites of lower animals that do not exist as saprophytes in nature and, depending on the species, rarely or frequently infect humans.

(3) *Geophilic* - parasites found free living in nature, with or without the ability to infect either animals and/or humans.

Examples of each type are listed in Table 1.3.

Table 1.3

Dermatophyte Epidemiology

Host Preference Type	Species
Anthropophilic	+ *Epidermophyton floccosum*
	Microsporum audouinii
	M. ferrugineum
	Trichophyton concentricum
	+ *T. mentagrophytes var interdigitale*
	+ *T. rubrum*
	T. schoenleinii
	+ *T. tonsurans*
	T. violaceum
Zoophilic	+ *M. canis*
	T. equinum
	T. gallinae
	+ *T. mentagrophytes var mentagrophytes*
	T. verrucosum
Geophilic	*M. cookei*
	M. fulvum
	+ *M. gypseum*
	+ *M. nanum*
	M. persicolor
	T. ajelloi
	T. gloriae
	T. terrestre

\+ examined in this study

Source: The Epidemiology of the Dermatophytes by L. Ajello in (3)

Dermatophyte infections are known by several different names, both common and specific, depending on the location of the infection. For example ringworm is the common name for localised skin infections usually due to *Microsporum canis*, whilst Athlete's foot (Tinea pedis) an infection of the foot, is usually caused by *Trichophyton mentagrophytes* and nail infections may be due to *T. rubrum*.

Tinea capitis, Tinea pedis, Tinea barbae, Tinea faciei, Tinea corporis, Tinea manuum, Tinea cruris and Tinea unguium describe superficial fungal infections of various regions of the body, often caused by more than one different fungal species (82, 128, 146). The world wide geographic distribution of the Dermatophytic fungal species has recently been reviewed by Philpot (142), who also lists the dominant species for the different infection areas in each geographical area.

1.5.12 Biochemistry

Despite the importance of the dermatophytes as human parasites, relatively little is known about their biochemistry and chemotaxonomy. The fatty acid and sterol content of representative species have been investigated (11, 24, 178, 179, 184) and found to be similar to those found in most other fungal species (80, 181). Of the sterols present, ergosterol is the most prevalent with significantly smaller quantities of brassicasterol, except in the *Microsporum* species examined (24). The sterol concentration varies from as little as 0.16% ergosterol in *M. canis* to 1.5% of dry mycelium in *T. persicolor* (24).

The fatty acid content is similar to most other fungi except for the widespread absence of fatty acids of chain length greater than C-18 and the absence of γ-linolenic acid (18.3) (178, 179, 181). The predominate fatty acids are linoleic, oleic, palmitic and stearic acids (178).

The cell wall composition of dermatophytes has been the subject of a number of studies (132, 133, 159), and the presence of chitin has been established by chemical analysis and X-ray diffracton. The precise quantities of chitin and the composition of the rest of the cell wall is not well defined due largely to the difficulties in analysis of such a complex structure.

1.5.13 Current Treatments for Dermatophyte and related Infections

Before the development of modern drug therapy the treatment of fungal diseases was restricted largely to the use of various topical creams and solutions, most of which have been discarded as being of little benefit in all but the most mild infections. These included gentian violet, Aquafor, ammoniated mercury ointment, benzoic acid (180), magenta paints, Pragmatar ointment and sulfur ointments (146). Taplin (168) has recently reviewed the development of treatment methods for superficial mycoses.

When considering treatment options for human fungal infections it is important to remember that diagnosis of the causal organism may be imperfect and that secondary infections of different organisms may be present. Since the treatment of many of these infections, particularly the deep seated and systemic infections, is an imperfect art, the medical practitioner frequently arrives at a successful treatment by trial and error.

Treatment for dermatophyte infections is limited to a few useful remedies. In most cases the preferred form of treatment for superficial dermatophytic infections involves the use of topical creams; including various forms of zinc undecylenate/undecylenic acid (widely used to treat Tinea pedis (athlete's foot), Tolnaftate (150) (Tinaderm), Clotrimazole (Canesten), Miconazole (Daktarin) and benzoic acid compound ointment BNF (Whitfield's ointment). The relatively recent introduction of Tolnaftate, Clotrimazole and Miconazole in topical creams has greatly improved the effectiveness of such treatments.

If these treatment methods are unsuccessful then the only recourse is to use the antimycotic Griseofulvin taken orally and has its effect as a result of being laid down in newly formed layers of skin. Griseofulvin only inhibits the growth of *Microsporum, Epidermophyton* and *Trichophyton* species (except *T. tonsurans* and *T. violaceum*), and large doses are required, up to 1gm/day for periods of up to one year. Because Griseofulvin only inhibits the growth of the fungus, the treatment must be continued until the infected tissue is shed.

The recent introduction of the animal antihelminthic agent Thiabendazole* (149) as an antidermatophytic agent may offer some improvement in the treatment of superficial dermatophyte infections.

* Registered trade mark

In some infections, particularly 'athlete's foot' combined steroid and antiseptic treatment (elioquinol hydrocortisone ointment) may bring some relief if there is 'weeping' associated with the infection (180).

Control of superficial *Candida* infections is usually effected by the use of topical applications of the polyene antibiotic Nystatin (Nystan) (often containing other polyene antibiotics such as neomycin, gramicidin and cortisones such as hydrocortisone), or Amphotericin B (Fungilin). Newer preparations including clotrimazole (Canestan), natamycin (Pimafucin), candicidin (Candeptin) and miconazole (Dakyarin) may be used topically in place of nystatin. In the case of an excess of *Candida* spp in the gut requiring treatment, oral doses of Nystatin or some of the other polyenes may be given.

The non-dermatophyte superficial fungal infections may be treated with a number of different drugs. Pityriasis versicolor (tinea versicolor) caused by *Malassezia furfur* is usually treated with selenium sulfide creams and solutions or sodium thiosulfate.

For severe superficial and systemic fungal infections as well as systemic candida infections the choice of treatments is limited to intravenous Amphotericin B or oral doses of 5-flurocytosine.

There are several excellent reviews of antifungal agents and their clinical use, including those of D'Arcy and Scott (61), Cartwright (48), Loeffler (118), Drouhet (65), Warin (180), Smith (161), Holt (91), Kobayashi and Medoff (107), and Winfield (183) while the adverse reactions to some of these drugs have been documented by Pascher (140).

1.6 Polyacetyelenes

The term polyacetylene is usually extended to include all polyyne molecules containing one or more acetylenic (-C≡C-) bonds. Naturally occuring polyacetylenes have been extensively investigated by Bohlmann and his co-workers (30) and over 600 different compounds have been isolated from a large number of plant species. Polyacetylenes have been isolated from a large number of species in the plant families Compositae and Umbelliferae whilst less extensive surveys have revealed the presence of polyacetylenes in 18 other plant families (see 1.6.2) as well as in some algae and micro-organisms (30).

For the purposes of this thesis only the straight chain hydrocarbon acetylenes that occur in plants and contain two or more acetylenic bonds will be considered in depth. For details of the chemistry and chemotaxonomy of other polyacetylenes the reader is referred to Bohlmann, Burkhardt and Zdero (30), and to Ross (150) for a review of the non-polyacetylenic acetylenes. The earlier review of polyacetylenes by Bullock (46) and the recent brief review of natural acetylenes by Jones and Thaller (102) also provide useful introductions to the isolation, identification and chemotaxonomy of naturally occuring acetylenic compounds.

Straight chain hydrocarbon polyacetylenes of varying chain length are widespread in the plant families Compositae and Umbelliferae and to date are the major polyacetylenes found in the Araliaceae and Pittosporaceae. These compounds are believed to be derived primarily from straight chain fatty acids by dehydrogenation from adjacent carbon atoms, followed usually, but not always, by the loss of the carboxylic acid function (see Fig 1.1). They are typically found in low concentrations (Table 1.4) and are often detected by spectroscopic methods, particularly by U.V. spectroscopy.

Table 1.4

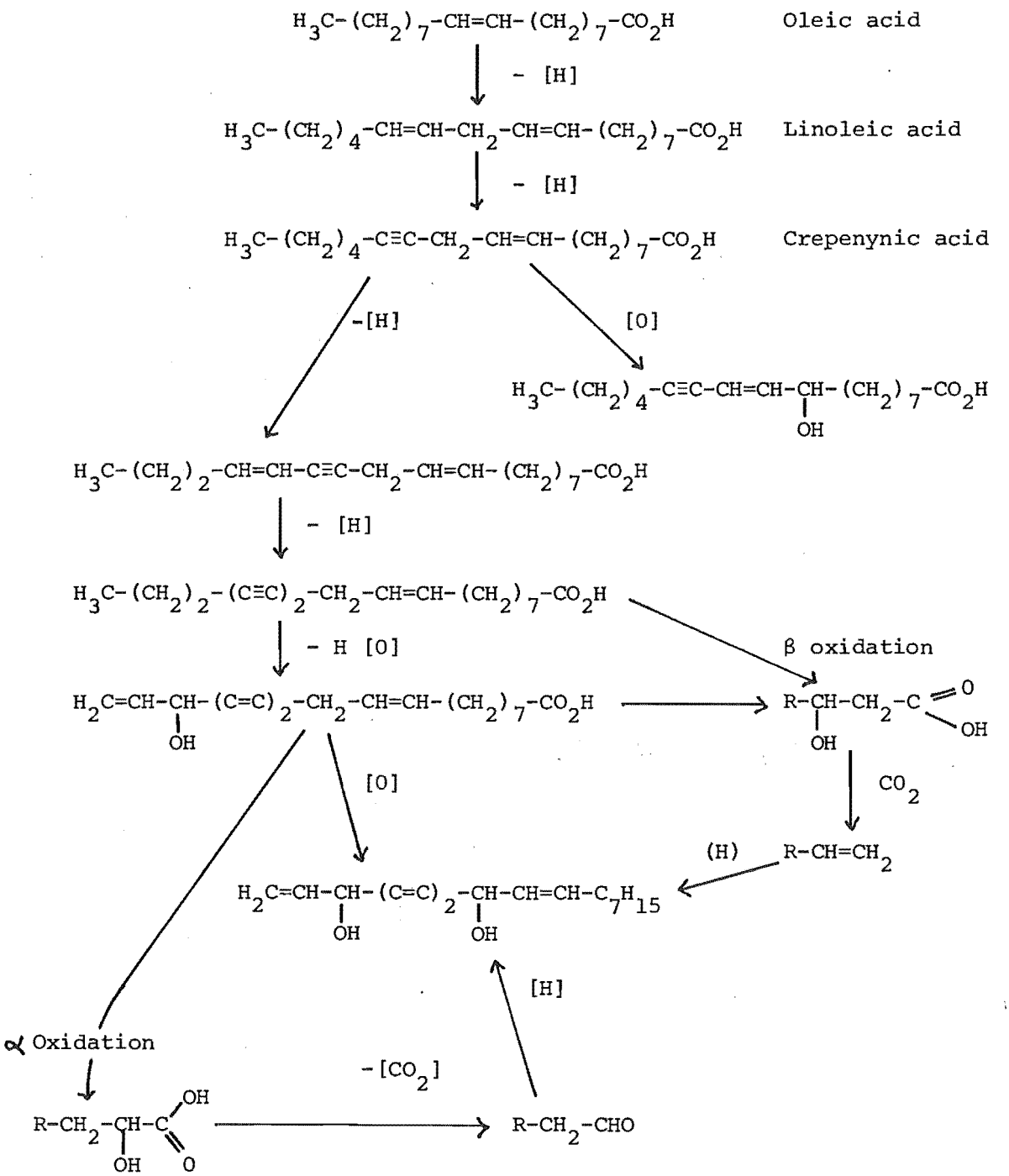
Levels of Polyacetylenes in Plant species

Species	Total Polyacetylenes ppm Fresh weight	Falcarindiol ppm fresh weight
Daucus carota (15)	37	5
Aegopodium podagraria (104)	250	210
Apium graveolens (27)	10	3
Falcaria vulgaris (32)	2700	5
Oenanthe crocata (34)	120	-
Opopanax chironium (33)	130	2.5
Carthamus tinctorius (7)	36	-

Simple polyacetylenes give highly characteristic U.V. spectra with sharp vibrational fine structure (Table 1.5). U.V. spectroscopy is often used to screen for polyacetylenes but this suffers from a serious disadvantage, often not recognised, that the intensity of the spectrum is proportional to the degree of conjugation, particularly with respect to any double bonds present. Simple mono-acetylenes, di and tri-acetylenes without further conjugation are virtually impossible to detect by U.V. spectroscopy (10, 30, 102). Thus it is possible that

Fig 1.1

Biosynthesis of C-17 and C-18 Polyacetylenes



Source: Bohlmann, Burkhardt & Zdero (30).

For further details of the biosynthesis of polyacetylenes see (30).

Note: None of the enzymes responsible for the above reactions have been identified.

Table 1.5

U.V. Spectra of Polyacetylenes

Compound	λ_{max}					ε_{max}				
$CH_2=CH-\underset{\underset{OH}{|}}{CH}-(C\equiv C)_2-\underset{\underset{OH}{|}}{CH}-CH=CH-C_7H_{15}$ falcarindiol	232	244	258			400	400	200 [a]		
$CH_2=CH-\underset{\underset{O}{\|}}{C}-(C\equiv C)_2-\underset{\underset{O}{\|}}{C}-CH=CH-C_7H_{15}$	242	253	268	285	303	14200	12500	10400	8900	6100 [b]
$CH_2=CH-\underset{\underset{O}{\|}}{C}-(C\equiv C)_2-CH_2-CH=CH-C_7H_{15}$	208	246	260	274	290	18400	3800	6400	10750	9550
$CH_2=CH-\underset{\underset{OH}{|}}{CH}-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_7-CH_2OH$	231	243	256	269	285	1048	953	782	664	540 [a]
$HO-CH_2-CH=CH-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_7-CH_2OH$	228	240	253	267	283	4900	6600	9300	13200	10600
$H_3C-CH=CH-(C\equiv C)_2-CH=CH-CH_2OH$	235	263	274	291	310	27500	7600	14400	21000	16900
$H_3C-CH_2-\underset{\underset{OH}{|}}{CH}-CH=CH-C\equiv C-(\overset{t}{CH=CH})_2-(CH_2)_3-CH_3$	295	311				35700	32500 [c]			

Interpretative notes:
a. weak spectra characteristic of interrupted chromophore
b. intensification of spectra due to extensive conjugation of the ketone
c. intense spectra of multiple double bond conjugation.

the presence of significant quantities of polyacetylenes with interrupted chromaphores and/or alcohol functions, such as falcarindiol, may have gone undetected in many earlier screening procedures. The U.V. spectral intensity of a number of different polyacetylenes is listed in Table 1.5.

1.6.1 Biologically active Polyacetylenes

The precise role of the polyacetylenes in plants has yet to be established so it is perhaps premature to divide polyacetylenes into those with biological activity and those without. In the present context the term 'biologically active' should therefore be qualified further as "antibiotic activity".

A number of naturally occuring and synthetic acetylenes have been shown to possess *in vitro* activity against a range of bacteria and fungi. Thomas and Allen (6, 7, 170) have isolated a number of polyacetylenes from Safflower, some of which possessed pronounced antifungal activity against phytopathogens such as *Phytophthora drechsleri*. They demonstrated (7) a forty fold increase in the concentration of the polyacetylene safynol in infected tissue.

Kawazu *et al* (103) have isolated from the Araliad *Dendropanax trifidus* two C-18 antifungal polyacetylenes with pronounced activity against *Cochliobolus miyabeanus*. During the last year Kemp (104) isolated falcarindiol from *Aegropodium podagraria* and demonstrated antifungal activity against a number of plant pathogens but did not test for activity against bacteria or human pathogenic fungi. Schulte (153) isolated a number of C-13 polyacetylenes possessing bacteriostatic and fungistatic activity.

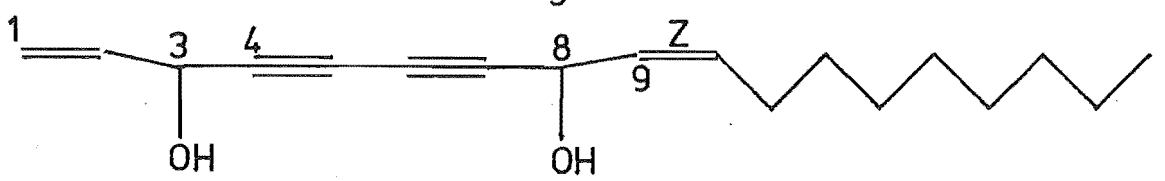
Tanaka (166), Imai (96) and Fawcett *et al* (74) have isolated and synthesised a number of polyacetylenes incorporating six membered rings with pronounced activity against plant pathogens, as in the case of Wyerone (74) or against dermatophytes in the case of Capillin and its derivatives (96, 166), while Reisch, Spitzner & Schulte (145) have tested 90 different polyacetylenes against ten different micro-organisms including six dermatophytes.

Towers *et al* (172) in their investigations of U.V. mediated photodermatitis have shown that a number of polyacetylenes possessed antibiotic activity against a range of bacteria and fungi, while others possess activity when irradiated with U.V. light.

1.6.2 Falcarindiol (9Z -heptadeca-1, 9-diene, 4-6-diyn-3, 8-diol)

This biologically active polyacetylene is a C-17 diacetylene (Fig 1.2)

Fig 1.2



(9Z)-heptadeca-1,9-diene-4,6-diyn-3,8-ol

and has previously been identified in a number of species belonging to the Umbelliferae (Table 1.6). The present study is the first reported isolation of falcarindiol from an Araliad.

Table 1.6

Reported Sources of Falcarindiol

Family: Umbelliferae

Species	Reference
Aegopodium podagraria	Kemp (104)
Apium graveolens L	Bohlmann (27)
Capnophyllum dichotomum	Bohlmann et al (30)
Crithmum maritimum	" " (30)
Daucus carota	Bentley et al (15) Bohlmann et al (32)
Eryngium alpinum L	Bohlmann & Zdero (36)
E. coeruleum LK	" " "
E. giganteum M.B.	" " "
Falcaria vulgaris	Bohlmann et al (32)
Heteromotpha trifoliata	Bohlmann et al (30)
Molopospermum cicutarium DC	" " (30)
Opopanax chironium	Bohlmann & Rode (33)
Pituranthus tortuosus	Schutte & Potter (156)

Falcarindiol was first isolated from *Falcaria vulgaris* Bernh. by Bohlmann *et al* (32) and the structure confirmed by Bentley *et al* (15) when isolating polyacetylenes from *Daucus carota* L. Subsequently, falcarindiol has been isolated from several other species.

There are several C-17 acetylenes closely related to falcarindiol, but with different functional groups at carbons 3 & 8 which have been isolated from a much larger range of plant species (30). All of these compounds (known as falcarinone types) have a diyne group with double bonds β to the diyne system. The intervening α carbons contain at least one and often two oxygen containing functions. There are also C-17 compounds differing from falcarindiol and related compounds only in that the saturated side chain is terminated with a vinyl double bond.

There is also a third group of polyacetylenes possessing what might be called a falcarinone structure. These are C-18 diynes where the saturated side chain terminates with an additional carbon bearing either an alcohol or a carboxylic acid function. (Table 1.7).

Table 1.7

Polyacetylenes related to Falcarindiol

	$CH_2=CH-CH(OH)-(C\equiv C)_2-CH(OH)-CH=CH-C_7H_{15}$	Falcarindiol
A	$CH_2=CH-CH(O-C(=O)-CH_3)-(C\equiv C)_2-CH-CH=CH-C_7H_{15}$	
	$CH_2=CH-C(=O)-(C\equiv C)_2-CH_2-CH=CH-C_7H_{15}$	Falcarinone
	$CH_2=CH-C(=O)-(C\equiv C)_2-C(=O)-CH=CH-C_7H_{15}$	Falcarindione
	$CH_2=CH-C(=O)-(C\equiv C)_2-CH(OH)-CH=CH-C_7H_{15}$	Falcarinolone
	$CH_2=CH-CH(OH)-(C\equiv C)_2-CH_2-CH=CH-C_7H_{15}$	Falcarinol
B	$CH_2=CH-C(=O)-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_5-CH=CH_2$	Dehydrofalcarinone
C	$CH_2=CH-CH(OH)-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_7-CH_2OH$	
	$CH_2=CH-CH-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_7-CH$	

1.6.3 Chemotaxonomy of Polyacetylenes

Acetylenic compounds have been isolated from a range of plant species as well as several fungal species (particularly Basidiomycetes) (90). Acetylenes have also been isolated from a few algal, yeast and bacterial species. Only two plant families however, have been investigated thoroughly; the Compositae and the Umbelliferae, where in excess of 1400 species, representing over 360 genera, have been reported to contain acetylenes (30). Since Bohlmann's authoritative book was published in 1973, extraction of polyacetylenes from other plant and fungal species has continued with acetylenes being found in a further 230 plant species (Table 1.8) as well as several fungal species (Appendix B).

Table 1.8

Recent reported isolations of Acetylenes

Plant Family	Number of species	Reference
Compositae	212	See Appendix A
Umbelliferae	18	(31, 37, 39, 40, 52, 139, 154, 156, 176)
Pittosporaceae	4	(38)
Araliaceae	1	(103)
Olacaeae	1	(124)
Solanaceae +	1	(76)
Leguminosae +	2	(147)
Campanulaceae	4	(12, 16, 17)

\+ first reported isolation from this family

The isolation of 3-hydrox-7, 8 de-hydro-β-ionol from *Nicotiana tabacum* (Solanaceae) (76) and the isolation of the furanoacetylenes wyerone and wyerone epoxide from *Lens culinaris* (Leguminosae) (147) bring to 21 the number of families from which acetylenes have been isolated.

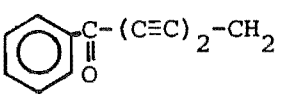
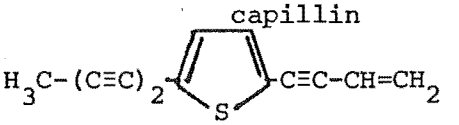
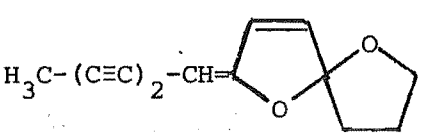
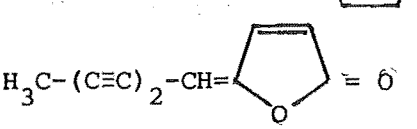
From the 21 families containing acetylenes the Compositae have been thoroughly investigated, and the Umbelliferae to a lesser extent, while of the remaining 18 families only about 100 species representing 49 genera have been reported to contain acetylenes. It is difficult to assess exactly how many plant species have been investigated with negative results, for example many other species of Compositae have been investigated by Bohlmann and his co-workers without finding any acetylenes present.

Nevertheless there are a few noticeable trends in the distribution of acetylenes within the genera investigated to date, sufficient for Bohlmann (30) to propose a model for the relationship of 12 families. The presence or absence of any particular acetylene is not however a very useful taxonomic guide except in the case of a few compounds with very restricted occurrences. However a few general observations can be made. The Umbelliferae, Pittosporaceae and Araliacae have as their most common acetylene the C-17 falcarinone (Table 1.7) and/or its many closely related compounds. As yet there have been no reported occurrence in these three families of polyacetylenes containing three or more conjugated triple bonds such as are found extensively in the Compositae. The Compositae, on the other hand, include species containing a wide variety of different polyacetylenes ranging from the widespread, simple highly unsaturated pentaynene and tetraynenes (Table 1.9) to the thiophene acetylenes found in the Inuleae, Heliantheae, Anthemideae, Arctotideae and Cynareae. The C-17 falcarinone type polyacetylenes are confined to a few tribes such as the Astereae, Heliantheae, Cotula and Artemisia, whilst thioethers, lactones and spiroketalenolethers have been isolated from a considerable number of species.

Thus, although there is a close relationship between the Umbelliferae, the Araliaceae and certain genera of the Compositae, the often simultaneous occurrence of other types of polyacetylenes in the Compositae must set them apart from the other two families.

Table 1.9

Types of Polyacetylenic compounds found in Plants

Pentaynene	$H_3C-(C\equiv C)_5-CH=CH_2$
Falcarinone	$CH_2=CH-\underset{\underset{O}{\|}}{C}-(C\equiv C)_2-CH_2-CH=CH-C_7H_{15}$
Aromatic acetylenes	
	capillin
Thiophene	
Spiroketalenolether	
Lactone	

2.0 EXPERIMENTAL

2.1 Isolation and Identification

2.1.1 Collection of Plant Material

Schefflera digitata ('seven finger'or'Pate') was collected from the following locations in the South Island of New Zealand:

(a) Ashley Gorge (Canterbury)

(b) Otira (West Coast)

(c) Fox Glacier (South Westland)

during the months of February, March, May and November.

Pseudopanax crassifolium and *P. colensoi* were collected from Lake Ianthe State Forest (Westland), *P. colensoi*, *P. simplex* and *P. lineare* were collected on the Denniston Plateau north of Westport. *P. arboreum* was collected from a Christchurch garden.

Prior to extraction the fresh material was separated into leaf lamina, petiole, stalk and inflorescences.

Voucher specimens are held at the University of Canterbury's herbarium.

2.1.2 Preparation and Preservation of Plant Material

All plant material not required for immediate extraction was freeze-dried, then ground to a coarse powder, and stored in sealed containers away from light. Oven-dried material was found to contain insignificant levels (undetectable by bioassay) of the antifungal compound falcarindiol and extracts failed to give a positive spray reaction (2.1.442 and 2.1.443) when subjected to TLC. For a comparison between fresh extraction, air-dired and freeze-dried material see 3.11.

Levels of activity have been expressed in one of two ways, Fresh Weight Equivalents (F.W.E.) or Dry Weight Equivalents (D.W.E.). The F.W.E. represents the measured fresh weight of the sample determined, if possible, within three hours of collection. The D.W.E. represents the weight after freeze drying.

2.1.21 Relationship of F.W.E. and D.W.E.

Table 2.1

	F.W.E.	D.W.E.
S. digitata	100	22.3
old leaves (inc petioles)		
Young leaves (inc ")	100	23.2
Young leaves	100	31
Mixed aged leaves	100	21
Petioles	100	17

In relating yields of the compounds present in *S. digitata* the following relationship has been used F.W.E. = (D.W.E.) x 5. Air dried material represents approximately 36% of the Fresh weight.

2.1.3 Extraction

Two bulk extraction methods were used. Initially prior to the identification of the active principle the ground material was extracted twice with ethyl acetate (10ml solvent per gm dry weight) at 4°C for 24 hours. The extracts were recovered by filtration and concentrated to a known volume.

After the identification of the active compound as falcarindiol all subsequent crude extracts were obtained by continuous extraction in a soxhlet extractor with diethyl ether. No increase in activity occurred after 15 hours of extraction. The resulting crude extracts were concentrated by evaporation under reduced pressure at 30°C using a rotary evaporator.

2.1.4 Separation Techniques

2.1.41 Gel Chromatography

Separation by gel permeation chromatography was performed on column's of Sephadex LH-20 using either 15% or 30% ethanol in water as the eluting solvent. The polysaccharide-based Sephadex gel was later replaced by the new Merck product Fractogel PGM-2000 (140-230 mesh), a polyethylene glycol dimethacrylate gel which, since it was designed for gradient elution in organic solvents, gave better separation than did Sephadex LH-20.

2.1.42 Column Chromatography

Column chromatography was carried out in glass column's of various diameters. Packing materials used were 10% deactivated alumina or Florisil (100-200 US mesh) (Fisons). All column's were packed as a slurry in hexane. Extracts were applied in petroleum ether or hexane in order to retain chlorophyll's and the active material on the column while the carotenoids were eluted.

2.1.43 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on 0.25mm layers of Silica Gel G or Silica Gel GF_{254}. Preparative layer chromatography was carried out on Silica Gel $PF_{254+366}$ (1.0mm layers). The TLC plates were not activated by heating prior to use since breakdown of the active material occurred on activated plates. The chromatograms were developed in diethyl ether,petroleum ether (3:7) unless stated otherwise. The compounds present were visualised by spraying with isatin (2.1.443) or phosphomolybdic acid (2.1.442).

2.1.44 Visualisation Agents

Four methods were available for detecting the presence of falcarindiol and related compounds on TLC plates. Of the four listed below, phosphomolybdic acid (2.1.442) proved to be the most useful despite the non-specific nature of the reaction. The heated chromatograms retained the resultant colour for several weeks and the reagents are relatively inexpensive. Isatin (2.1.443) was slightly more sensitive and gave an immediate colour response, but suffered from the disadvantage of containing conc H_2SO_4. U.V. examination was much less sensitive and required double indicator TLC plates. Nitrobenzyl-pyridine (2.1.444) is expensive and offered no advantage over isatin or phosphomolybdic acid.

2.1.441 Ultra-Violet Examination

TLC plates were examined under U.V.Light (254nm) for any distinctive fluorescence. Falcarindiol appeared as a pale blue spot when chromatographed on double indicator plates (Silica gel containing $F_{254+366}$ indicators.). The main use of this technique was in locating the active compound on preparative TLC plates as the technique is (largely) non-destructive.

2.1.442 Phosphomolybdic Acid

Falcarindiol appeared as a distinctive black spot when sprayed with a 4% solution of phosphomolybdic acid (Sigma) in ethanol. The colour developed only after heating to 100°C.

2.1.443 Isatin

Falcarindiol appeared immediately as a dark brown spot when sprayed with a 0.4% solution of isatin in conc H_2SO_4 (59).

2.1.444 Nitrobenzyl-pyridine

A 5% solution of 4-(4'-nitrobenzyl)-pyridine (N.B.P.) in acetone was sprayed on fluorescent TLC plates. The colour was intensified by spraying with 10^{-5}M NaOH (152).

2.1.5 Derivatives

Infrared spectroscopy of the unknown compound (3.4.3) indicated that alcohol functions were present, and since parent compound was not sufficiently volatile for glc analysis nor very stable, derivatives of the alcohol were made. It was hoped that the derivatives would be more stable and possibly crystalline. It was also anticipated that the presence of recognisable functional groups such as TMS or acetate would aid in the identification of the compound.

2.1.51 Tri-methyl Silane Derivative

The TMS derivatives were prepared by dissolving the compound in 1ml of dry pyridine (distilled over KOH), to which 0.4ml of HMDS (1, 1, 1, 3, 3, 3, 3, 3, 3-hexamethyl disilazane, Aldrich) and 0.2ml of TMCS (trimethyl chlorosilane, Sigma) were added. The mixture was allowed to stand for one hour, reduced to dryness and taken up in pentane. The derivative was purified by filtration through a small cotton wool column and subjected to glc to determine the number of components present.

2.1.52 Acetate Derivative

The acetate derivative was prepared by dissolving 20mg of the purified active compound in 2ml of pyridine and 2ml of acetic anhydride. The reaction proceeded for 18 hours and was stopped by addition of 20ml of water. The acetate was extracted from the aqueous solution by

diethyl ether (4x 10ml), washed with 10% Na_2CO_3 (3x 20ml), then with distilled water (2x 20ml), and dried with Na_2SO_4 prior to concentration. The acetate was further purified by preparative TLC.

2.2 Determination of Biological Activity

2.2.1 Test Organisms

The following dermatophytes were used as test organisms:

Microsporum gypseum
M. canis
M. nanum
Trichophyton rubrum
T. mentagrophytes
T. mentagrophytes var interdigitale
T. erinacei
T. tonsurans
Epidermophyton floccosum

Other organisms tested were:

Bacillus subtilis
Escherichia coli
Candida albicans
Pseudomonas sp.
Staphylococcus aureus
Aspergillus niger
A. fumigatus
Botrytis cinerea
Fusarium oxysporum.

2.2.2 Culture Methods

The dermatophytes and yeasts were regularly sub-cultured on slopes of Sabouraud agar (Difco Neopeptone 10g/L, glucose 40g/L, agar 1.5%). Bacteria were maintained on nutrient agar (B BL) (N.A.) and the fungi on potato dextrose agar (PDA). The cultures were grown in 25°C incubators. (See 3.1.23).

2.2.3 Antibiotic Assay Disc Bioassay

Two different methods were used to prepare assay discs for this bioassay method.

2.2.31 Semi-quantitative Method

This was used to test crude extracts and column fractions for biological activity. The extracts were made up to a standard volume in glass vials. Sterile 6mm antibiotic assay (AA) Disc's (Whatman)

were dipped into the solution, drained and transferred to the surface of agar plates seeded with spores of the appropriate test organism.

2.2.32 Quantitative Method

This method was used to test pure compounds and to quantify activity in crude extracts. Known volumes of the test solutions were transferred to the sterile AA discs using disposable micropipettes or microsyringes. The dry discs were then transferred as in 2.2.31.

Bioassay plates were incubated for 48-72 hours at 27°C. The diameter of the resulting inhibition zone was recorded in mm. Prior to the identification of the active compound, the standard bioassay solvent was ethanol; but after identification of the compound as falcarindiol all subsequent bioassay samples were prepared in diethyl ether.

2.2.4 Slide Germination Method

The effects of falcarindiol on the germination of dermatophytes was observed using cavity slides containing 0.05ml of spore suspension. The spore suspension was prepared by washing the spores from agar slopes with Sabouraud broth. This spore suspension was then filtered through two layers of sterile muslin to remove any mycelial fragments, and diluted to approximately $1.5\text{-}2.0 \times 10^5$ spores per ml.

The slides were incubated in a humidity chamber (saturated atmosphere) at 20°C and examined under the optical microscope at regular intervals for three days to determine the percentage germination. Germination was taken to be the visible appearance of a germ tube from the spore. (See 3.3.2). Significant germination of the dermatophytes was only obtained when the spore suspension was prepared in Sabouraud broth.

2.3 Microscopy

2.3.1 Optical Microscopy

For optical microscopy the spores were germinated in cavity slides (2.2.4) and exposed to varying concentrations of the test compounds. The material was observed live using either direct lighting or phase contrast microscopy.

2.3.2 Transmission Electron Microscopy (TEM)

The material for TEM examination was grown in liquid culture for 36 hours. The spores were centrifuged into a loose pellet using a bench centrifuge (1000g) and this concentrated spore suspension transferred with a sterile pasteur pipette to small aluminium foil boats containing molten 1.5% water agar at 40°C. The agar was then allowed to set and the resulting block fixed according to the following schedule.

The agar blocks containing the spores were fixed in 6.5% glutaraldehyde in 0.1M Sodium cacodylate buffer, pH 7.6 for 12 hours at 4°C (119), followed by post-fixation in 2% osmium tetroxide (OsO_4) for 4 hours at 4°C. The specimens were dehydrated in ethanol, washed twice in propylene oxide, infiltrated and embedded in Araldite resin. Thin sections were cut on a LKB ultrotome using a glass knife and examined in a Hitachi HS-7 transmission electron microscope.

3.0 RESULTS

3.1 Preliminary Screening of Crude extracts

Initial screening of crude extracts confirmed earlier observations of biological activity against dermatophytic fungi made by Bong (41) and Lee (114). These tests indicated that an antibiotic compound(s) could be extracted with most common solvents. The highest levels of activity were obtained when organic solvents were used, whilst water extraction gave little or no activity against the dermatophytes *T. rubrum* and *M. gypseum*. (Table 3.1)

Table 3.1

Solvent extraction of Antifungal Activity

Solvent	*T. rubrum*				*M. gypseum*			
	Diameter of inhibition zone (mm)							
	30	10	5	1mg/ml	30	10	5	1mg/ml
Diethyl-ether	17	15	13	9	15	12	10	8
Ethyl acetate	14	13	9	-	12	10	8	-
Petroleum ether	12	11	8	-	11	10	8	-
Chloroform, Methanol (1:1)	12	11	10	-	10	8	-	-
Methanol	13	11	9	-	8	8	-	-

using Semi-Quantitative method (2.2.31)

Three different procedures were tried to extract the active compound from the plant material. Of these only the solvent extraction method (2.1.3) resulted in a biologically active extract. Steam-distillation and solvent extraction at elevated temperatures failed to yield biologically active extracts.

The crude extracts were tested for antibiotic activity against a range of micro-organisms confirming the initial observations of Bong (41) that the dermatophytic fungi were much more sensitive to the inhibitory action of the active compound from *S. digitata* than other fungi. These initial experiments were repeated in more detail using purified falcarindiol in place of the crude extracts (3.3).

3.11 Evaluation of Plant Preservation Methods

Four methods of plant tissue preservation were investigated for their effects on the yield of the antifungal polyacetylene falcarindiol. These were fresh material, freeze-dried, air-dried and oven-dried leaves. Of these four methods the oven dried material appeared devoid of any falcarindiol (by bioassay and TLC). The three remaining preservation methods were compared for their effects on extraction efficiency by bioassay and TLC. 100g F.W.E. of each sample was extracted with diethyl ether in a soxhlet extractor and concentrated to 1ml = 5g F.W.E., Antibiotic assay discs impregnated with the crude extracts (2.2.32) were tested for activity against *M. gypseum*. All three extracts exhibited distinct inhibition zones at the 25mg F.W.E./ disc level. Below this concentration some differences were apparent with air-dried extracts failing to produce a zone of inhibition below 12.5mg F.W.E. (cf M.I.C. for fresh and freeze dried extracts of 5mg F.W.E./disc).

Thin Layer Chromatography of all three crude extracts revealed zones co-chromatographing with falcarindiol in each case. There were however, distinct differences between extracts in respect of the other compounds present. The fresh extracts contained significantly fewer compounds capable of reacting with the phosphomolybdic acid reagent (2.1.442) than did the freeze-dried extracts.

Freeze drying was chosen as a means of preservation for the bulk samples rather than using fresh material despite the presence of a more complex mixture of compounds present because (a) collection of material was only possible on infrequent occasions, (b) Freeze dried material was less bulky and therefore more material could be extracted at once, and (c) the presence of water in the crude extracts of fresh material meant that the crude ether extracts had to be dehydrated prior to further purification.

Extracts prepared from plant material collected at three different locations (2.1.1) at different times of the year, contained similar levels of antifungal activity indicating that there were not marked seasonal or locational differences in the production of falcarindiol.

3.12 Evaluation of Bioassay Methods

3.121 Effect of Spore Concentration on Inhibition Zone Diameter

In order to test the effect of variation in spore concentration on inhibition zone diameter, agar plates were prepared and seeded with a range of spore concentrations of the dermatophyte *T. rubrum*. Falcarindiol impregnated AA discs (10 to 80 µg/disc) were placed on the agar surface and the plates incubated at 27°C. After 72 hours the zones on the lowest spore concentration plates were noticeably larger, but this difference had largely disappeared after 96 hours.

Subsequently all measurements of inhibition zones were obtained by measuring zones on consecutive days after the appearance of a continuous mycelial mat, since the precise level of spore inoculum appeared to have little effect on the size of the inhibition zone. All inhibition zone measurements on fungal plates were obtained in this way. Bacterial bioassay's were measured after 24 and 48 hours' incubation.

3.122 Effects of Solvents on Inhibition Zone Diameter

Solvents with high boiling points (e.g. higher alcohols) tended to inhibit the growth of the dermatophytic fungi and were avoided. The solvents used (diethyl-ether, hexane, chloroform and ethanol) had no detectable effect on spore germination and subsequent hyphal growth.

The effect of the solvent on the size of the inhibition zone was tested by adding a standard quantity (25 µl) of solvent to AA discs impregnated with different quantities of falcarindiol after they had been placed on the agar surface. There was no enhancement of the inhibition zone due to the presence of excess solvent (Table 3.2).

Table 3.2

Falcarindiol µl/disc	Diameter of inhibition zone (mm)+	
	Control	+25 µl Diethyl ether
10	10.3	10.6
20	12.0	10.6
40	13.3	13.0
80	14.3	15.0

\+ average of three replicates
test organism: *M. gypseum*

Placing sterile discs on the agar surface and then adding the test compound to the discs with a micropipette produced similar inhibition zone diameters indicating that the solvent was not having any effect on the inhibition zone diameter.

3.123 Effect of Incubation Temperature on Antibiotic Activity

The effect of various incubation temperatures on the diameter of the inhibition zones was investigated. Because dermatophytes colonising human skin occupy an environment where the mean temperature would be in the range 28-37°C, a range of temperatures up to 40°C were investigated (Table 3.3).

Table 3.3

Temperature	Diameter of inhibition zone (in mm)+				
	5	10	25	50	µg falcarindiol/disc
25°C	8	10	14	18	
30°C	8	11	14	19	
37°C	10	16	17	21	
40°C	No spore germination				

+ mean of three replicates
Test organism: *M. gypseum*

These results indicated that there was no significant temperature effect on the diameter of the inhibition zone, and therefore the effect of falcarindiol on *M. gypseum* spore germination is independent of temperature. All subsequent bioassays were incubated at between 25°C and 27°C.

3.2 Screening of Plants related to *S. digitata*

Crude extracts were made of several species of *Pseudopanax* since they were also Araliads and related closely to *S. digitata*. The extracts were subjected to both TLC and biological assays, the results of which are given in Table 3.4.

Table 3.4

Antibiotic Activity in New Zealand Araliaceae

	Inhibition Zone Diameter (mm)					
	Extract - Mg Dry Wt/Disc					
Test Organism = *Microsporum gypseum*	20	40	80	100	200	300
Schefflera digitata ("Seven Finger")	9	10	12	15	16	N.T.
Pseudopanax lineare	-	-	-	-	-	-
P. simplex	-	-	-	-	-	8
P. colensoi	-	-	-	-	-	-
P. arboreum ("Five Finger")	-	-	-	-	-	8
P. crassifolium ("Lance Wood")	-	-	-	-	-	-
Test Organism = *Bacillus subtilis*						
S. digitata	9	13	14	14	15	N.T.
P. lineare	-	-	-	-	7	9
P. simplex	-	-	-	-	9	10
P. colensoi	-	-	-	-	-	-
P. arboreum	-	-	-	-	-	-
P. crassifolium	-	-	-	-	-	8

This experiment indicated clearly that antibiotic activity was confined to *S. digitata* and that only nominal levels of falcarindiol could have been present in the related *Pseudopanax* species.

Examination of the crude extracts by TLC revealed the presence of a large number of compounds but none of them co-chromatographed with falcarindiol or gave positive colour reactions for polyacetylenes when the plates were sprayed with either isatin or phosphomolybdic acid (2.1.4).

3.21 Extraction of *Pseudopanax arboreum*

A further large scale extraction of *P. arboreum* ('five finger') was carried out in order to check for trace levels of polyacetylenes. 100g of freeze-dried leaves were extracted in a soxhlet with diethyl ether, yielding 12.9g of crude extract. The crude extract was examined by TLC on silica gel, revealing a number of compounds possibly common to *S. digitata* but none of them co-chromatographed with falcarindiol.

3.2g of this crude extract (25g DWE) was treated with charcoal to remove chlorophyll. The non-absorbed material was washed from the charcoal with diethyl ether and concentrated to a small volume. On cooling a large quantity of white crystals formed which were filtered off for further analysis. The remaining liquor was evapourated to

dryness under reduced pressure and dissolved in n-hexane prior to chromatography on a column of Florisil (185 x 8mm-10ml). The components of the extract were eluted step wise with increasing quantities of diethyl ether (ether,hexane 1:9, 3:7, 1:1 and finally diethyl ether alone). The fractions were subjected to TLC (ether,hexane, 3.7) and the plates visualised with phosphomolybdic acid. None of the fractions contained a compound which co-chromatographed with falcarindiol.

The crude extract was bioassayed for activity against *M. gypseum* over the concentration range. 12.5 to 250mg D.W.E. but no antibiotic activity was detected (cf. *S. digitata* M.I.C. 2.5 to 5mg F.W.E.-500 times lower concentration).

3.3 Specificity of Antibiotic Action

A range of micro-organisms were tested for susceptibility to falcarindiol in three assay systems; A.A. disc assay, cavity slide germination and liquid culture.

3.31 Antibiotic Disc Assay

A large number of microbial species were tested using this method (Table 3.5). Both crude extracts and pure falcarindiol were assayed. Microscopic examination of the clear inhibition zone indicated that no spore germination had occurred within the zones observed on the dermatophyte plates. Different isolates of several of the dermatophytes were tested with no significant difference between isolates being apparent.

3.32 Slide Germination Test

The slide germination method was used to examine the effect of falcarindiol on the germination of a range of fungi (Table 3.6). This bioassay method also allowed observation of the effects of falcarindiol on the spores. The results of this experiment confirmed the observations of the previous experiment (3.31) except in the case of *A. fumigatus*. However *A. fumigatus* appeared to be resistant to similar concentrations of falcarindiol when tested in liquid culture (3.33).

Table 3.5

Antibiotic activity of Falcarindiol as determined by the Antibiotic Disc Assay

	Diameter of inhibition zone (mm)							
Falcarindiol µg/disc	10	20	40	80	10	20	40	80
Organism	Disc applied before germination				Disc applied after germination			
Microsporum gypseum	14	14	21	22	7	8	9	9
M. canis	17	20	25	28	10	11	19	20
M. nanumm	20	21	25	26	11	12	13	13
M. gypseum	12	15	17	18	8	8	9	9
M. gypseum	14	16	20	21	8	8	9	10
Trichophyton rubrum	14	15	18	21	8	10	10	10
T. rubrum	14	16	18	21	8	8	9	10
T. erinacei	9	10	12	14	11	12	12	13
T. tonsurans	16	17	21	23	N.T.	N.T.		N.T.
T. mentagrophytes	14	19	22	25	9	10	12	12
T. mentagrophytes	10	10	13	16	8	8	9	9
T. mentagrophytes	11	13	20	22	8	10	11	12
T. mentagrophytes var interdigitale	12	15	17	19	N.T.	N.T.		N.T.
Epidermophyton floccosum	18	22	23	25	11	12	12	13
Non dermatophytes								
Bacillus subtilis	12	12	14	16	-	7	9	10
B. subtilis	8	9	13	14	-	-	7	9
Escherichia coli	-	-	-+	-+	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-
Pseudomonas sp	-	-	-	-	-	-	-	-
Staphylococcus aureus	9	11	13	14	-	-	-	-
Aspergillus fumigatus	-	-	-	-	N.T.	N.T.		N.T.
Verticillium dahliae	-	-	-	-	N.T.	N.T.		N.T.

N.T. not tested
+ pronounced solvent effect

Table 3.6

Antibiotic Activity of Falcarindiol as determined by the Slide Germination Test

Organism	% Germination			
	Falcarindol µg/ml			
	0	50	100	250
Botrytis sp.	100	80	60	50
Aspergillus fumigatus[1]	60	10	0	0
A. niger	60	50	50	30
Cladosporium resinae	100	80	60	50
Fusarium oxysporum	100	80	10	10
Microsporum gypseum[2]	60	0	0	0

1 see Table 3.7. These experiments were performed simultaneously
2 dermatophyte

3.33 Liquid Culture

The effect of falcarindiol on the growth of a number of fungi in stationary liquid culture was also investigated. This bioassay method was tried to ensure that the inhibition observed in the other bioassay systems were not artifacts. The stationary liquid culture method was chosen for its simplicity and also to see if falcarindiol could be dispersed in an aqueous environment and still retain its biological activity (for the effect of shaking see 4.3).

Spore suspensions (as for 3.32) were added to sterile test tubes containing falcarindiol or diethyl ether as a solvent control. The tubes were incubated at 25°C and observed for a period of 14 days. Each tube was scored on the basis of visual mycelial growth (Table 3.7).

Table 3.7

Antibiotic Activity of Falcarindiol in Liquid Culture

Organism	Falcarindiol µg/ml 0	50	100
Botrytis sp.	+++	+++	+++
Aspergillus fumigatus	+++	+++	+++
A. niger	+++	+++	+++
Cladosporium resinae	+++	+++	+++
Fusarium oxysporum	+++	+++	+++
Colletotrichum sp.	+++	++	++
Microsporum gypseum[1]	+++	-	-
M. nanum[1]	+	-	-
Trichophyton rubrum[1]	+++	-	-
T. mentagrophytes[1]	++	-	-
Eipidermophyton floccosum[1]	+++	-	-

+++ extensive mycelial growth, + minimal surface or submerged mycelial growth, - no visible mycelial growth
1 - dermatophyte.

3.4 Isolation of the Active Compound

The choice of a successful separation method is dependent upon a knowledge of the compound under study. In the beginning very little was known about the nature of the active compound so a range of different solvents were tested for their ability to extract the active compound (Table 3.1). Having established that several solvents could extract the active compound initial purification trials were based on the assumption that the methanol soluble fraction would separate by paper or gel chromatography since these separation methods posed the least problems for detecting the biological activity.

It soon became apparent that separation of the active compound from the chlorophyll and carotenoid components of the leaf extracts would be the major problem. Initial separation using gel chromatography (3.4.11) yielded an antibiotic compound, largely free of chlorophyll, but still containing most of the carotenoids. These fractions were purified further by paper chromatography (3.4.12) but it was not possible

to separate the active compound from carotenoids or carotenoid-like material.

Gel chromatography was capable of separating the active compound from chlorophyll; but the capacity of the column was limited to about 5g D.W.E. and difficulty was experienced in removing the chlorophyll from the column so that the expensive packing material could be re-used. Several attempts were made to separate the active compound from the crude extract by solvent partitioning. The active compound could be transferred from one organic solvent to another but only limited progress was made in partitioning the extract from aqueous methanol: NH_4OH solutions into hydrocarbon solvents. This approach was abandoned when the development of a TLC system lead to the determination of the chemical structure of the active compound.

Much of the initial work on the separation and identification of the active compound was frustrated by its apparent instability. The active compound was found to be unstable when exposed to air and to polymerise to a red, insoluble material when the purified extracts were taken to dryness. Storage of the compound was a problem until it was observed that storage in ethereal solution maintained the biological activity for at least one year. After the chemical structure had been elucidated, drying of extracts was avoided where ever possible in order to minimise the polymerisation.

3.4.1 Preliminary Purification Trials

3.4.11 Gel Chromatography

Initial separation was achieved on Fractogel PGM 2000 (2.1.43). Crude ethyl acetate extracts (5g DWE) were applied to columns (13 x 540mm) of PGM 2000 in isopropanol: 0.2N acetic acid (1:1). The column was eluted with the same solvent, the fractions freeze dried (to remove the isopropanol), taken up in ethanol and bioassayed. Antibiotic activity was eluted between fractions 10 and 17 (Fig 3.1) (2 to 4 bed volumes).

The active fractions were pooled and chromatographed on Whatman No 1 paper. The chromatograms were developed ascendingly in methanol, water (6:4). The zones active by bioassay (Rf 0.7-1.0) were recovered and pooled until 200mg of semipurified active compound was obtained. Some of this material was used for a number of separation trials (3.4.12) whilst the rest was further purified for ^{13}C-NMR spectroscopy (3.4.31).

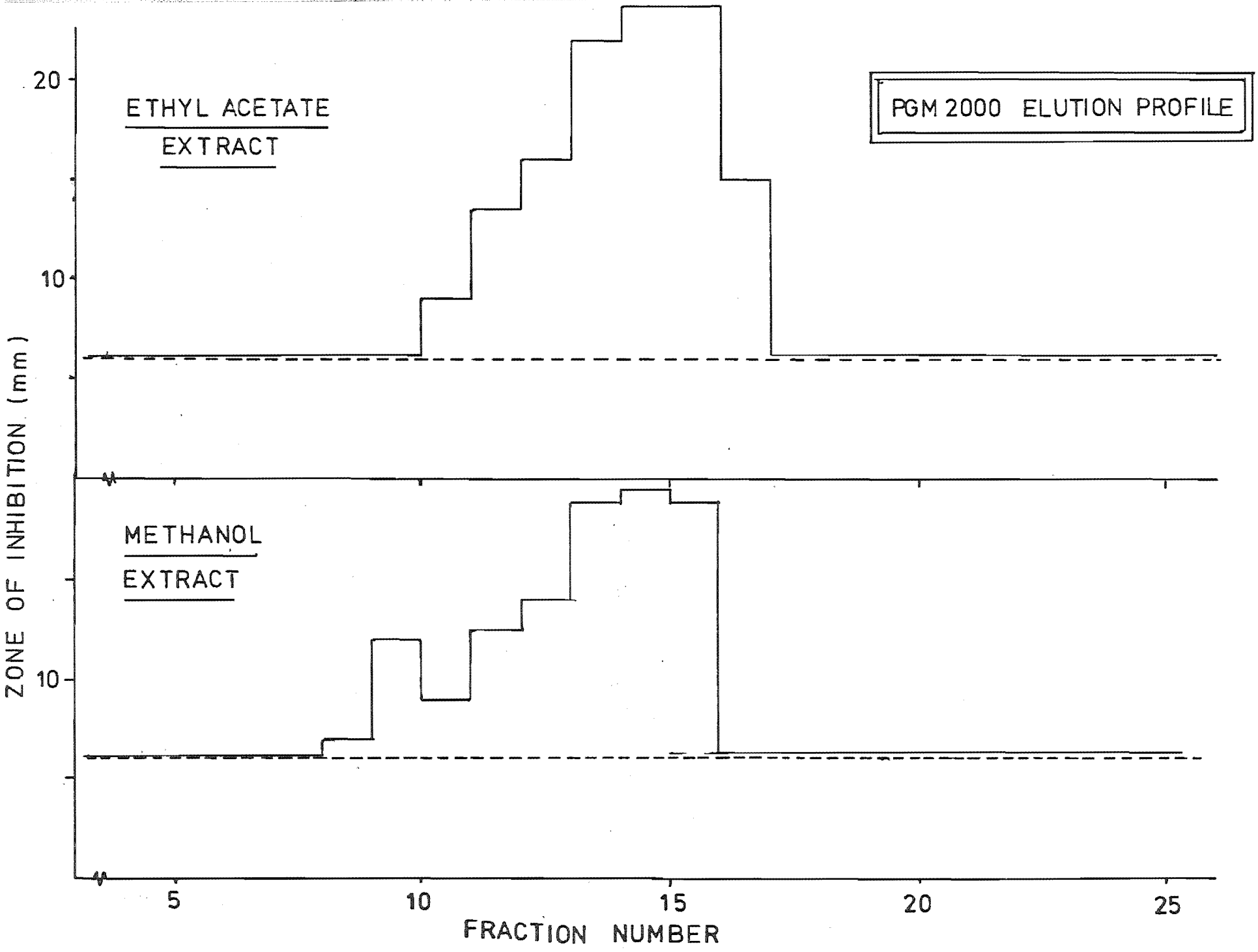

PGM 2000 ELUTION PROFILE
ETHYL ACETATE
EXTRACT
METHANOL
EXTRACT
ZONE OF INHIBITION (mm)
20
10
10
5
10
15
20
25
FRACTION NUMBER

Fig 3.1

3.4.12 Paper Chromatography

Paper chromatography was chosen initially since it was possible to bioassay directly from the paper. A large number of chromatographic solvents were tried (Table 3.8), however none of these proved totally satisfactory. All chromatograms were air-dried in a sterile environment prior to bioassay. All bioassays included controls which gave no solvent effects.

Table 3.8

Separation of the Active Compound by Paper Chromatography

Solvent	Active Zone (Rf)
2% Acetic Acid	0.0-0.2
Acetone,Water (7:3)	0.9-1.0
Benzene,Diethyl ether (9:1)	0.9-1.0
Benzene,Acetic Acid:Water (6:7:3 upper phase)	0.9-1.0
Butan-1-ol,Acetic Acid,Water (12:3:5)	0.7-1.0
Butan-1-ol,Acetic Acid,Water (4:1:5 upper phase)	0.8-1.0
Butan-2-ol, 25% Ammonia (4:1)	0.9-1.0
Butan-1-ol,Benzene,NH_4OH (50:2:48)	0.9-1.0
Chloroform,Ethyl acetate (1:1)	origin & 0.8-1.0
Chloroform,Hexane (1:1)	origin & 0.8-1.0
Ethyl acetate,Acetic Acid,Water (3:1:3)	0.9-1.0
Methanol,Water (3:1)	0.8-1.0
Methanol,Water (6:4)	0.7-1.0
Propan-2-ol,Acetic Acid,Water (60:30:2)	0.9-1.0
Propan-2-ol,0.2N Acetic Acid (1:1)	0.8-1.0
Propan-1-ol,1N Acetic Acid (3:1)	0.8-1.0
Propan-2-ol, 25% Ammonia,Water (8:1:1)	0.9-1.0

all solvents v/v
Whatmann No 1 paper

Recovery of the active compound from the paper for more detailed bioassay proved to be dependent upon the solvent used; of the solvents tested, methanol gave the best recovery, followed by ethyl acetate and chloroform. Water failed to elute any activity from the paper.

A number of common chromatographic spray reaction tests (Table 3.9) were tried in order to obtain some indication of the chemical nature of the active compound, however all tests gave negative or inconclusive results.

Table 3.9

Chromatographic Spray Reagents Tested

(all with negative results)

Test Reagent	Group Specificity
Sodium nitroprusside	S-S linkages
Dinitrophenylhydrazone	ketones/aldehydes
Folin-Ciocalteu reagent	phenols, etc.
Diazo spray	imidazoles
Ninhydrin	amino acids
Ammonium Fe sulphate	flavanoids
Sodium nitroprusside/NaOH	methyl ketones

3.4.13 Amberlite XAD-2 Resin

An attempt was made to use the novel ion exchange resin Amberlite XAD-2 (a non-ionic polymeric absorbant- co-polymer of styrene and divinyl benzene, 20-50 mesh, (Mallinckrodt) to purify the crude extracts prior to column and paper chromatography.

Two methods were utilised depending on the size of the extract. For small extracts the procedure of Fig 3.2 was used. The column was prepared according to Peltit *et al* (141). The crude extracts were taken up in 1N-NH_4OH, applied to the column, and the activity eluted with methanol. The resin was regenerated by washing with 2N acid and 2N alkali followed by a bed volume of acetone. Larger samples were treated in a batch process using the same solvents. Although this method gave a good separation of the active compound from the rest of the extract, considerable difficulty was experienced in dissolving the crude extracts in the 1N-NH_4OH, so the procedure was later superseded by chromatography on alumina columns.

3.4.14 Ultraviolet Spectroscopy

Most polyacetylenes are characterised by very distinctive U.V. spectra (30). Falcarindiol (the active compound) has a unique U.V. spectrum, but it is much less intense and distinctive than those of other polyacetylenes (Table 1.5). Determination of the presence of polyacetylenes in crude extracts containing chlorophyll or carotenoids was not possible in this instance because even the presence of trace amounts of carotenoids masked the less intense spectrum of falcarindiol.

Fig 3.2

Flow diagram for XAD-2 Resin

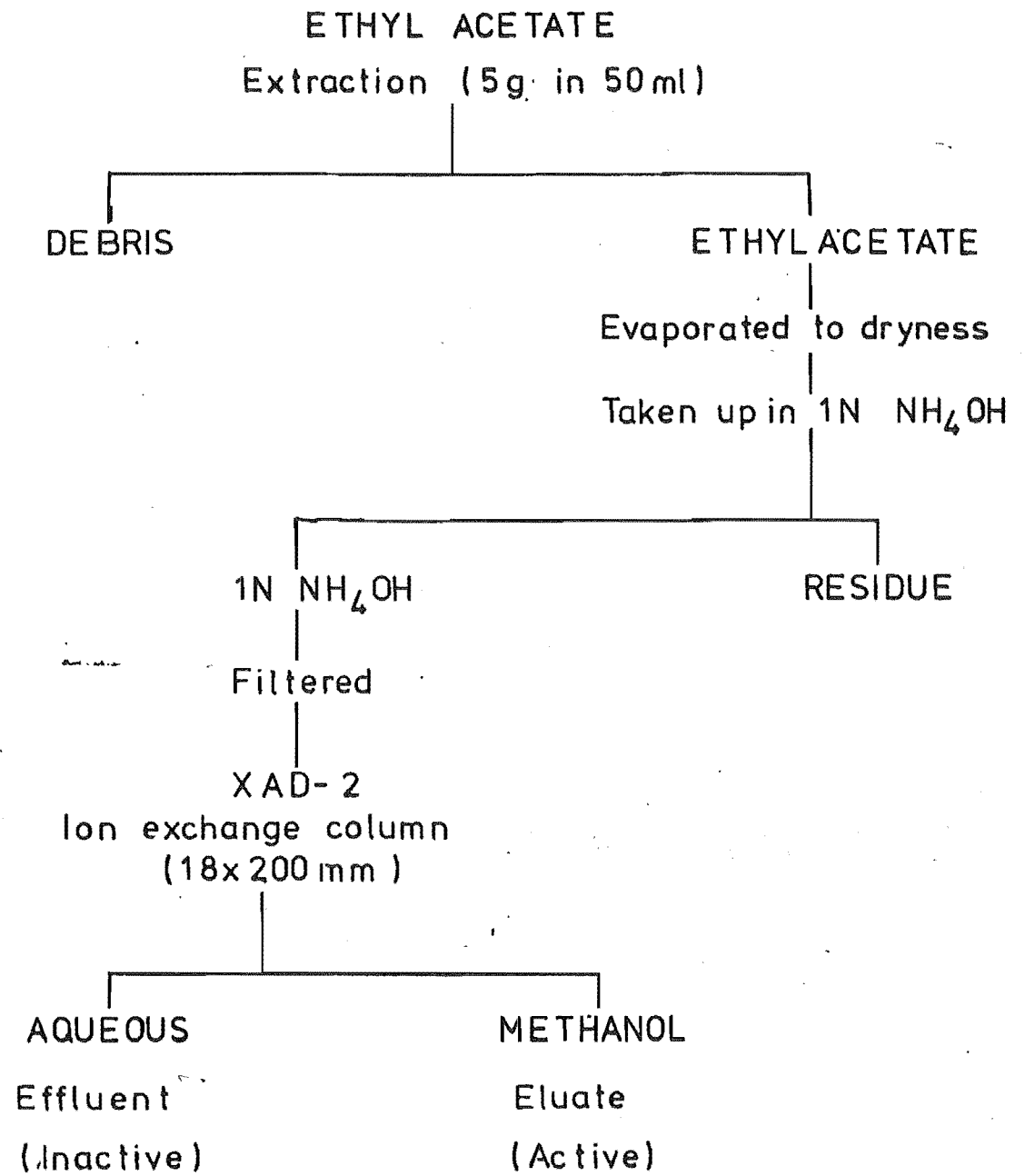


Table 3.10

U.V. Spectrum of Falcarindiol

Observed spectrum[1]							
A	λ_{max}		233	246	259		
	Relative E		1.0	1.0	0.7		
B	λ_{max}	224	233	246	260	275	291 nm
	Relative E	1.0	1.0	0.8	0.6	0.4	0.3
Literature (for falcarindiol (15))							
	λ_{max}	232	244	258 nm			
	ε	400	400	200			

1 obtained in diethyl ether
A spectrum obtained immediately after preparative TLC
B spectrum of same compound after a period of storage

Charcoal was used to remove the chlorophyll's prior to U.V. determination of extracts but there was not a 100% recovery of falcarindiol from the charcoal. Difficulty was also experienced in obtaining U.V. spectra because many ethereal solutions were found to give strong U.V. absorption in the region 250-290nm after having been taken to dryness prior to the determination of the U.V. spectrum. This effect could be largely eliminated by not allowing the solution to evaporate to dryness.

Thus the published ε_{max} for many polyacetylenes must be viewed with some suspicion because, for compounds like falcarindiol, the determination of the initial weight of compound, necessary for ε_{max} determination, must also result in some loss of the compound due to polymerisation.

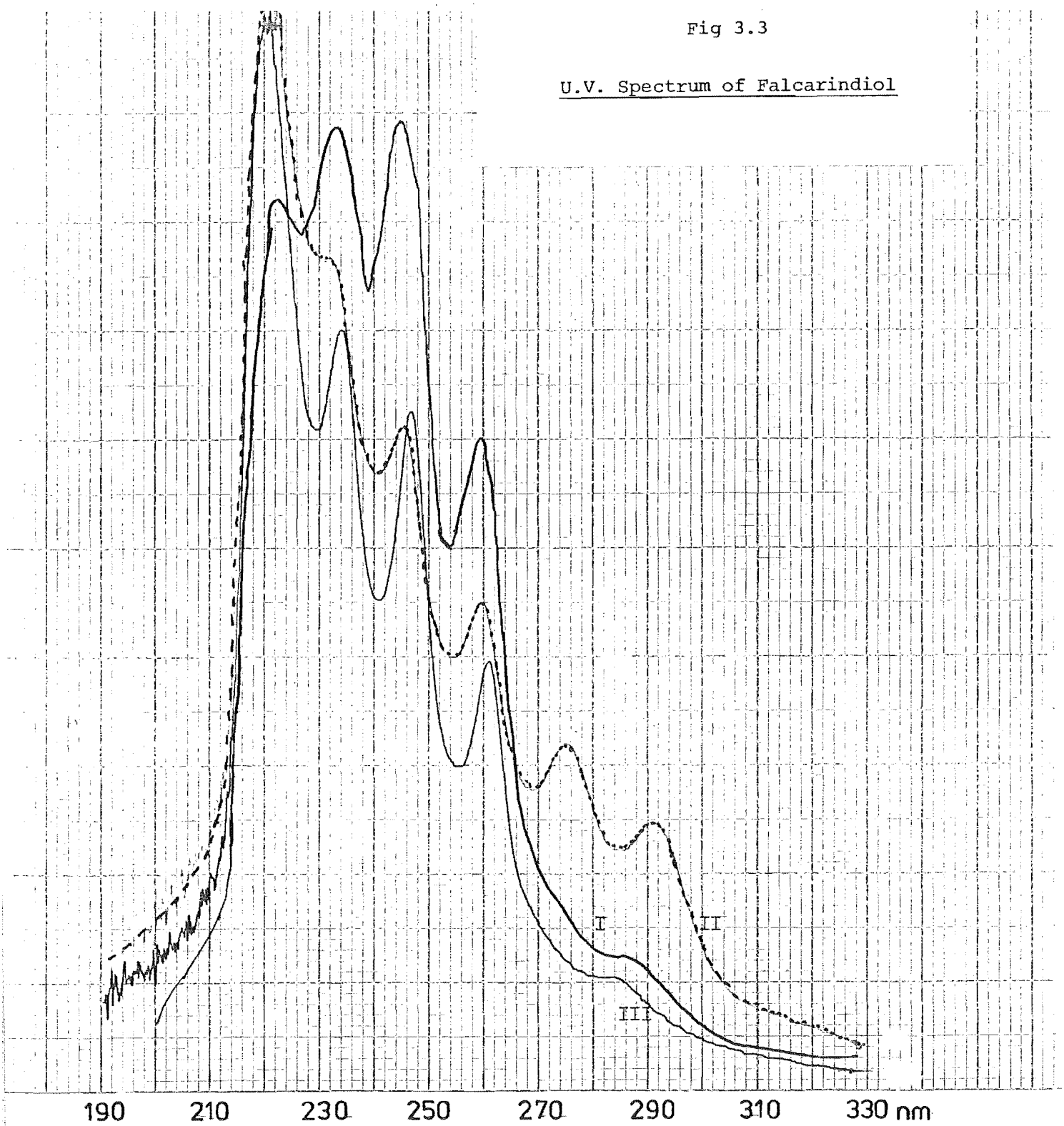
3.4.2 Isolation of Active Material

3.4.21 Column Chromatography

Separation of the active compound (falcarindiol) from the crude extracts was performed on a number of different alumina columns. The conditions for each column were varied slightly so as to optimise the separation of falcarindiol from the rest of the extract and to reduce the steps required. It was observed that the ratio of alumina to applied extract should be greater than 50.1 for optimum separation, although design of the column could also affect this ratio.

Fig 3.3

U.V. Spectrum of Falcarindiol



Sample	I	Falcarindiol (A)	Reference				
	II	Falcarindiol (B)				Slit 0.3mmm	
	III	Falcarindiol Diacetate	Solvent diethyl ether				
Scan speed	1.0	nm/sec x Chart speed	10	sec/cm=Expansion	10	nm/cm	
Absorbance range	190-330 nm	Path length	10 mm	Operator	A.D.Muir	Date	

Two distinct approaches to the problem of separation of falcarindiol were tried. Firstly; separation was attempted on a long narrow column (e.g. 17 x 140mm). Good separation was achieved but two technical problems were encountered. Firstly retention of the chlorophyll on the column (necessary for separation of falcarindiol from this group of compounds) only occurred when the extract was applied to the column in petroleum ether or hexane. This meant that large volumes (approximately one bed volume) of solvent were required to load the sample onto the column since the crude extract was less soluble in pure hexane or petroleum ether than in solvent mixtures containing diethyl ether, thus resolution was impaired.

The second problem encountered was the formation of air bubbles in the column packing material. Air was removed from the column packing by suspending it in solvent and placing it under vacuum until the evolution of bubbles stopped. This reduced the severity of the gassing problem but did not eliminate it entirely. As a consequence of this problem complete development of a column was protracted since air bubbles slowed the flow rate, and it was sometimes necessary to stop development which resulted in mixing occurring on the column. Using this technique it was difficult to separate falcarindiol from a chlorophyll type compound with a slightly lower R_f.

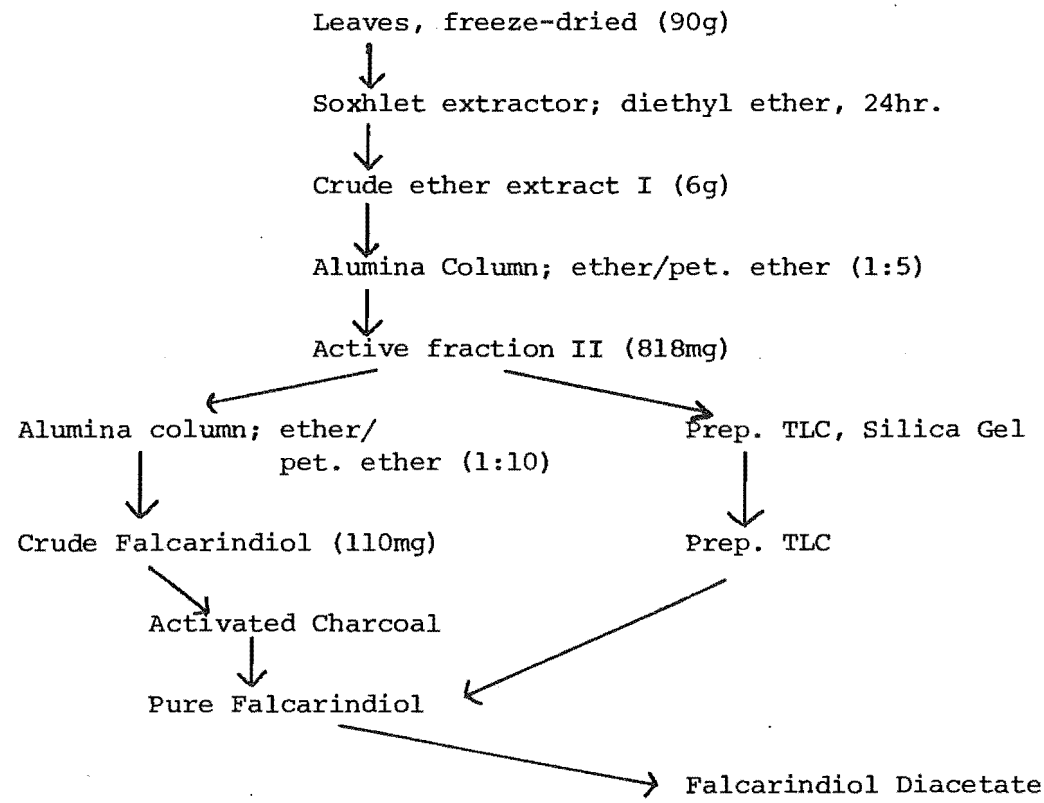
The most effective method of removing this contamination was to treat the extract with charcoal from which the falcarindiol was subsequently eluted with diethyl ether.

In later columns the ratio of diameter to length was increased from 1:8 to 1:2 and the number of steps in the gradient elution profile reduced with the result that larger quantities of crude extracts could be processed. Adequate separation was still achieved and the volume of solvent required reduced. It appeared that the best separation was achieved by changes in solvent composition (partitioning) rather than by relying on the physical properties (e.g. molecular filtration) of the column packing; therefore the shorter and wider the column the more concentrated would be the bands eluted.

Data for two columns are presented, the first was an early developmental column whilst the latter represents the final stage of development. (Many of the developmental columns were abandoned and therefore not fully documented). The flow diagram (Fig 3.4) summarises the purification procedure employed.

Fig 3.4

Isolation of Falcarindiol from *Schefflera digitata*



3.4.22 Developmental Alumina Column

6g of crude extract (approximately 90g D.W.E.) was applied to a column, containing 300g of 10% deactivated alumina, in 10% diethyl ether in petroleum ether. The column was eluted with the following solvent gradient: 700ml of diethyl ether;petroleum ether (1:10), 800ml of a 1:5 mixture and then 600ml of a 1:2 mixture: 10ml fractions were collected and the separation monitored by TLC. Fractions with similar TLC patterns were combined and tested for biological activity against *T. rubrum* (see Table 3.11).

A white crystalline material crystalised from fraction F, and was recovered by filtration. The crystals were purified by recrystalisation and subsequently identified as 1-hexacosanol (ceryl alcohol) (see 3.5.2).

Table 3.11

Chromatography of Crude extracts on Alumina

Fraction	Elution Vol. (ml)	Chromatographic Analysis (R_f)	Biological Activity
A	0-190 (=1 bed volume)		-ve
B	190-340	Carotenes 0.95-1.0	-ve
C	340-370	3 major components 1.0 0.88 0.8	-ve
D	370-440	2 " " 0.88 0.8	-ve
E	440-500	Several minor components 0.63 0.54 0.42 0.38	-ve
F	500-600	2 " " 0.42 0.38	-ve
G	601-650	2 " " 0.42 0.38	-ve
H	650-740	1 major 0.24 2 minor 0.42 0.38	-ve
I	740-780	1 major sport 0.24 + Trace 0.38	-ve
J	780-820	1 major component[1] 0.17 + 0.24 and Trace 0.38	+ve
K	820-980	1 " " 0.17	+ve
L	980-1100	Several minor components 0.17 and 0.09	-ve

1 identified as Falcarindiol

Fractions J and K were combined (818mg) and rechromatographed on a column containing 40g of 10% deactivated alumina in an effort to separate falcarindiol from the chlorophyll which had coeluted from the first column. The extract was applied in petroleum ether and development of the column begun with diethyl ether, petroleum ether (1:10). Falcarindiol was eluted from this column between 3 and 21 bed volumes. The ether content of the eluting solvent was raised to 20% half way through the development.

The yield of purified falcarindiol was 674mg with an average purity of 90% which represents approximately 0.7% of dry weight of plant material. From this a total of approximately 200mg of 99% pure falcarindiol was eventually recovered after treatment with charcoal.

A minor component present in this fraction was separated from falcarindiol and tentatively identified as phytol (3.5.3).

Fractions E to I were combined and a further 75mg of white crystals recovered. The remaining material was separated by preparative TLC (PLC) and seven compounds recovered, none of which gave positive polyacetylene reactions when TLC plates were spryaed with isatin (2.1.443). These compounds were recovered but no further investigations were carried out.

3.4.23 Preparative large scale column

200g of freeze dried material was extracted with diethyl ether yielding 15.5g of crude extract. The crude extract was taken up in hexane and applied to a column of 10% deactivated alumina (900g) (Vo=900ml:81x150mm). The column was eluted at a rate of 450ml/hr with the following solvent sequence: hexane 1.2L, diethyl ether: hexane (15:85) 1.4L, (3:7) 2.1L, ethyl acetate 2.0L. The column fractions were examined by TLC and grouped as in 3.4.22. (See Table 3.12).

Table 3.12

Solvent	Fraction	Volume (L)	Yield (mg)
Hexane	1	0.9-1.2	76
Hexane, diethyl ether (85:15)	2	1.2-1.7	213
"	3	1.7-1.9	730
"	4	1.9-2.0	52
"	5	2.0-2.3	65
"	6	2.3-2.8	139
Hexane, diethyl ether (7:3)	7	2.8-3.2	550
"	8	3.2-3.4	170

Table 3.12 (continued)

Solvent	Fraction	Volume (L)	Yield (mg)
Hexane,diethyl ether (7:3)	9	3.4-3.8	380
"	10	3.8-4.5	196
"	11	4.5-4.7	259
Ethyl acetate	12	4.7-5.9	1410
"	13	5.9-7.1	

TLC of these combined fractions indicated that falcarindiol was present in fractions. Fractions 11 and 12 were combined and estimated by TLC to contain 1200mg of crude falcarindiol which is 0.6% of the dry weight of material (or 0.12% of the fresh weight).

Fractions 11 and 12 were further purified by treatment with charcoal to remove any remaining chlorophyll, then subjected to PLC to give pure falcarindiol. The potential yield of falcarindiol was about 90% of the crude preparation, but in practice only 60 to 70% was actually recovered due to absorption on the charcoal and polymerisation on the TLC plates. The falcarindiol was recovered from the TLC plates as fast as possible to minimise the latter effect.

Fraction 10 (196mg) was subject to preparative layer chromatography in diethyl ether,hexane (2:3). This yielded 15mg of pure falcarindiol and 17mg of a mixture of falcarindiol and a second compound with a higher R_f.

Fractions 7 and 8 were combined (720mg) taken up in warm hexane, and then allowed to cool. The resulting white crystals were recovered by filtration for a total yield of 270mg of crystals from three crystalisations. (Ceryl alcohol).

3.4.22 Thin Layer Chromatography

TLC on silica gel plates proved to be the most convenient way of detecting falcarindiol. Using this method 205µg of falcarindiol could be detected using either the isatin or phosphomolybdic acid spray reagents. Two precautions had to be observed for satisfactory results. Firstly, the TLC plates must not have been activated by heating otherwise there was an unpredictable catalytic effect on falcarindiol, and secondly glass plates must be used since aluminium backed plates were highly active and effected the spray reactions.

The most suitable solvents were mixtures of diethyl ether and hexane or petroleum ether. The ratio was varied to achieve different separations.

3.4.3 Identification of the Active Principle

3.4.31 Experimental

The active compound was extracted and purified as described previously (3.4.2), with the biological activity of the purified extracts monitored at each purification step.

The Infra Red spectra were recorded in CCl_4 solution in a Shimadzu IR-27G spectrophotometer.

GLC separation was performed on OV-17 and SE-30 columns in a Varian Series 1400 Gas chromatograph equipped with F.I.D. Optimum separation of the TMS derivatives was obtained on OV-17 or SE-30 columns run isothermally at temperatures between 220°C and 240°C. Below these temperatures the retention time of the TMS derivative increased rapidly and sensitivity was lost.

The high resolution chemical ionisation mass spectrum (HRCIMS) was obtained on an AEl MS902 equipped with a SRI Cemspect CI Source (AEl) at School of Chemistry (University of N.S.W.), with 0.1% ammonia in methane as the ionizing gas.

The ^{1}H-NMR spectra (60MHz) were obtained in a Varian T-60 spectrophotometer using TMS as the internal standard ($\delta_{TMS} = 0.00$).

The ^{13}C-NMR spectra were obtained as CCl_4 solutions in a Varian CFT-20 instrument. The chemical shifts (δ_c) have been expressed as ppm downfield from the internal standard trimethylsilane (TMS).

Electron Impact mass spectroscopy was performed on two different machines. GC-MS (70ev) was carried out on an appropriately equipped AEI MS 32 mass spectrometer at the Mt Albert Research Centre, Auckland, while direct probe insertion M.S. (70ev) was carried out on an AEI MS 902 at the Chemistry Department, University of Canterbury.

The initial ^{13}C-NMR spectrum of the active compound was obtained on material prepared in the following way. Active fractions from several PGM-2000 columns (3.4.11) were combined, evaporated to dryness and dissolved in methanol. This active material was strip loaded on to 20 x 20 cm sheets of Whatman No 1 paper and developed in Methanol, H_2O (6:4) in the ascending mode. The active zones (by bioassay) were eluted from the paper with methanol and the combined eluates filtered through a Gelman 0.45 µm Millipore filter. The active

material was concentrated under vacuum and dissolved in CCl_4. This solution was further purified by chromatography on a small column of non-absorbant cotton wool which removed a yellow oily material present in the extracts.

All subsequent spectroscopy was carried out on material purified by PLC and TLC.

The following are the spectroscopic data for (9Z) - heptadeca-1,9-dien-4,6-diyn-3,8-diol (Falcarindiol). U.V. (Diethyl ether) λ_{max} 233 (Relative E 1.0), 246(1.0), 259(0.7)nm; I.R. (CCl_4) $\bar{\nu}_{max}$ 3610, 3400(OH), 3100, 3050(CH_2=CH-), 2240, 2150 (C≡C), 1860, 1650(CH=CH)cm^{-1}; ^{1}H-NMR ($CDCl_3$) ppm 0.90(3H,t,CH_3), 1.26-1.28(10H,m,$(CH_2)_5$), 2.07(2H, m,CH_2-C=), 2.97-3.6(2H,s,-OH), 4.97-5.58(7H,CH_2=CH-,CH=CH, and CH(OH) twice); ^{13}C-NMR (CCl_4) 135.98(d), 133.21(d), 128.24(d), 116.54(t), 79.58(s), 78.13(s), 70.30(s), 68.87(s), 62.84(d), 58.01(d), 31.70(t), 29.22(t), 29.05(t), 27.48(t), 22.51(t), 14.01(q).

(9Z) 3,8-diacetoxy-heptadeca-1,9-dien-4,6-diyne.
I.R.(CCl_4) 3100, 3050(CH_2=CH-), 2240, 2150(C≡C), 1860, 1650(CH=CH), 1745, 1200 (Acetate)cm^{-1}; ^{1}H-NMR(CCl_4) 0.88(3H,t,CH_3), 1.26(10H,m, $(CH_2)_5$), 2.00(3H,s,Acetate CH_3), 2.03(3H,s,Acetate CH_3), 1.6 & 2.2 (2H,CH_2-C=), 5.2-6.16(7H,CH_2=CH-,-CH=CH and -CH(OAc) twice); ^{13}C-NMR(CCl_4) 167.49(s), 135.53(d), 132.16(d), 124.16(d), 119.04(t), 76.36(s), 74.79(s), 70.60(s), 69.11(s), 63.67(d), 59.19(d), 31.63(t), 28.91(t), 27.54(t), 22.44(t), 20.24(q), 13.95(q); CIMS m/e 362.2380, M+NH_4^+. ($C_{21}H_{32}O_4N$ requires 362.2331), m/e 345.2081, M+H. ($C_{21}H_{29}O_4$ requires 345.2066), m/e 344.1994, (M+H)-H, ($C_{21}H_{28}O_4$ requires 344.1987.). For a detailed listing of the mass spectrum see Appendix C.

3.4.32 Interpretation

The purified compound was dissolved in CCl_4 and the I.R. spectrum obtained (Fig 3.5) revealed the presence of a sharp absorption at 3610cm^{-1} and a broad absorption at 3400cm^{-1} characteristic of -OH stretching and H-bonding. Dilution of the solution resulted in a decrease in the relative intensity of the 3400cm^{-1} absorption and an increase in the free -OH absorption at 3610cm^{-1} which indicated that intermolecular hydrogen bonding was occurring. Intense absorption bands at 2850 and 2900cm^{-1} indicated a saturated chain whilst two sharp absorptions at 2240 and 2150cm^{-1} indicated a possible acetylenic system.

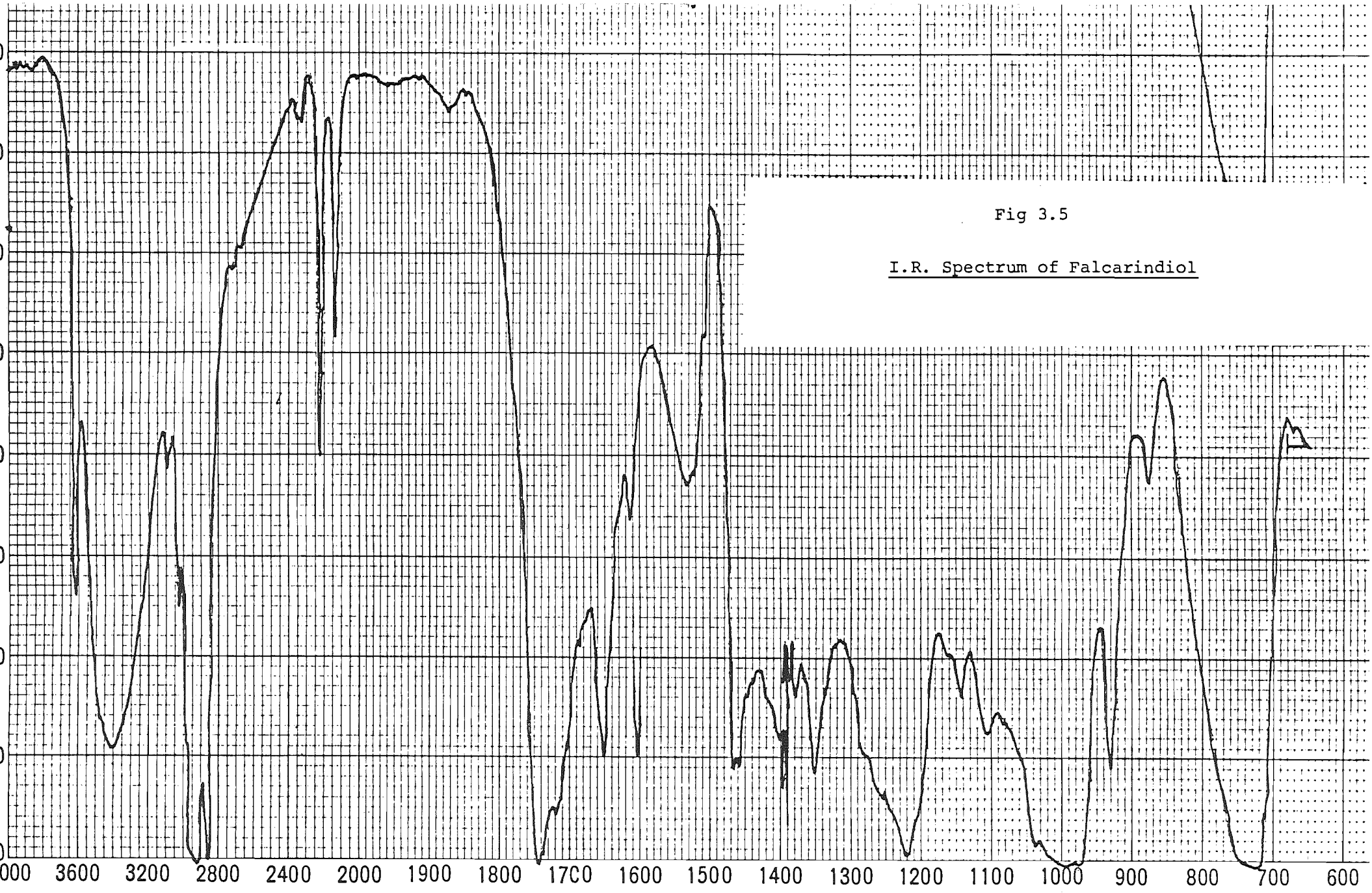


Fig 3.5

I.R. Spectrum of Falcarindiol

There was however, no absorption at 3300cm^{-1} due to any C-H stretching in the -C≡C-H system; thus any acetylenic system must be internal. Medium and weak absorptions at 1650 and 1860cm^{-1} respectively indicated a non-conjugated double bond, possibly symmetrically substituted, whilst absorptions at 3050 and 3100cm^{-1} due to the C-H stretching of vinyl double bond protons were present.

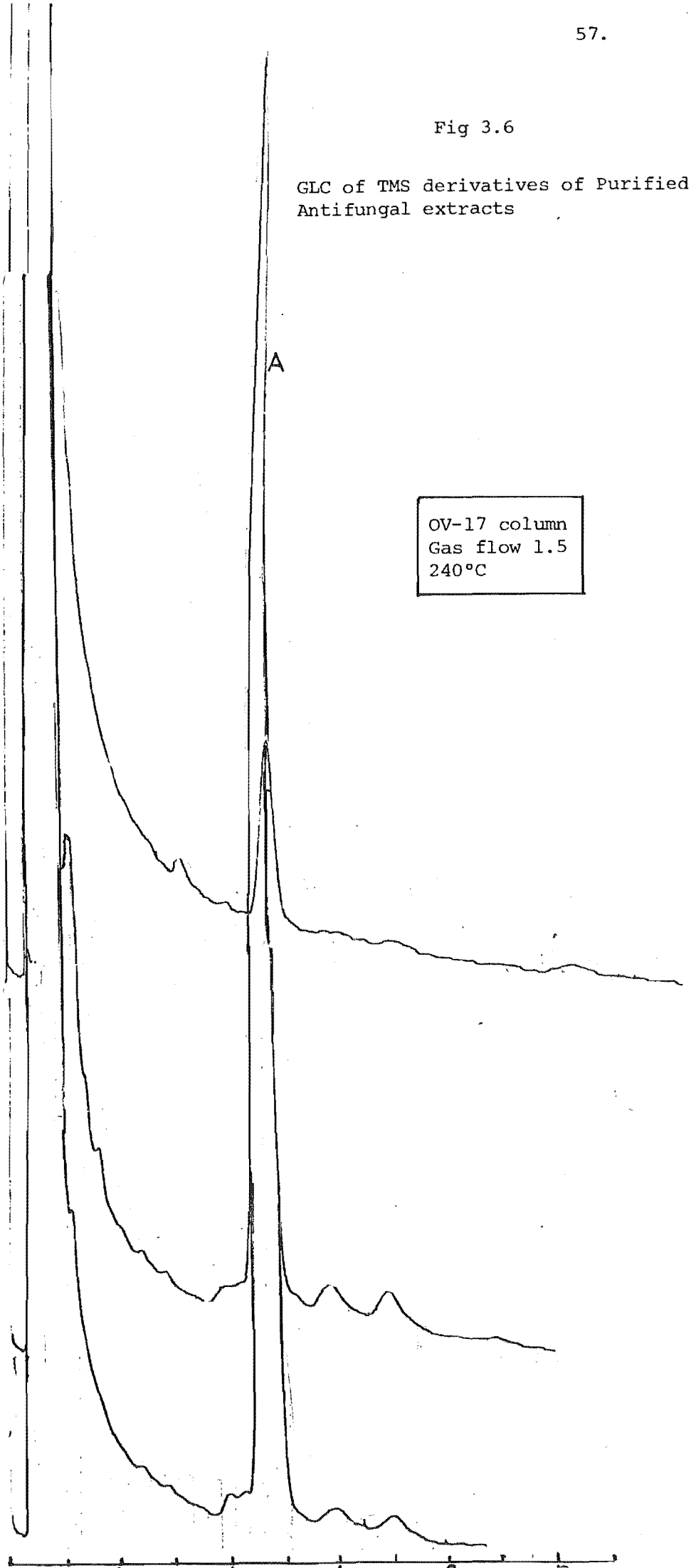
Gas liquid chromatography (glc) was used to check the purity of the active compound and determine its volatility. Initially glc on OV-17 columns were unsuccessful since the compound was not sufficiently volatile. However, since the I.R. spectrum indicated the presence of hydroxyl functions the trimethyl silyl derivative (TMS) was prepared (2.1.61) and subjected to glc (Fig 3.6). This derivative gave a single major peak (A) although there was a slight tendency for the TMS derivative to decompose over a period of days. Glc of TMS derivatives prepared from several different, biologically active, purified extracts all gave single major peaks with similar retention times (Fig 3.6). TMS derivatives prepared from a number of other biologically active extracts revealed the presence of two further peaks with greater retention times than A. (Fig 3.7). These peaks B and C were also observed if the TMS derivative was reduced to dryness and re-silyated. It was presumed that they represented either re-arrangements due to migration of the TMS group or a molecular re-arrangement due to traces of methanol present in most of the purified extracts at that time.

The electron impact mass spectroscopy of the TMS derivatives (3.4.5) failed to give a molecular ion or recognisable fragments although GC/MS did result in a mass spectrum which could be interpreted once the identity of the compound had been established (see 3.4.5).

The TMS derivative did not appear to be a suitable derivative for identification and, since it was unlikely to possess antifungal active, the acetate derivative was prepared (2.1.62). In the I.R. spectrum of the acetate derivative (Fig 3.8) the absorptions at 3610 and 3400cm^{-1} had disappeared and were replaced by absorptions at 1745 and 1200cm^{-1} due to the acetate group. This derivative was also sufficiently volatile to be analysed by glc.

Fig 3.6

GLC of TMS derivatives of Purified Antifungal extracts



N.B. These extracts did not give peaks B & C when chromatographed on SE-30 (Fig.3.7)

Fig 3. 7

GLC of TMS derivatives
of Antifungal extracts

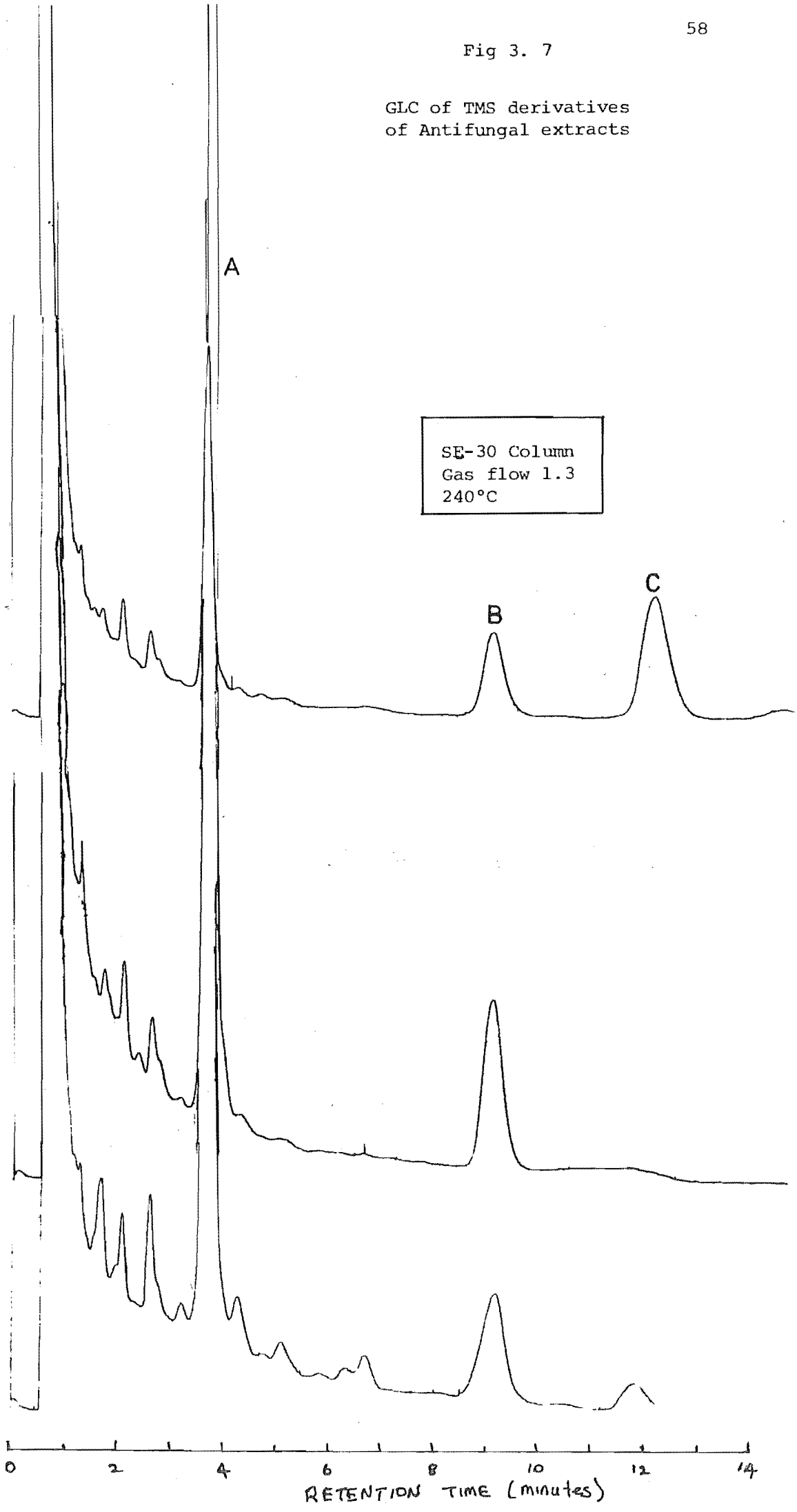
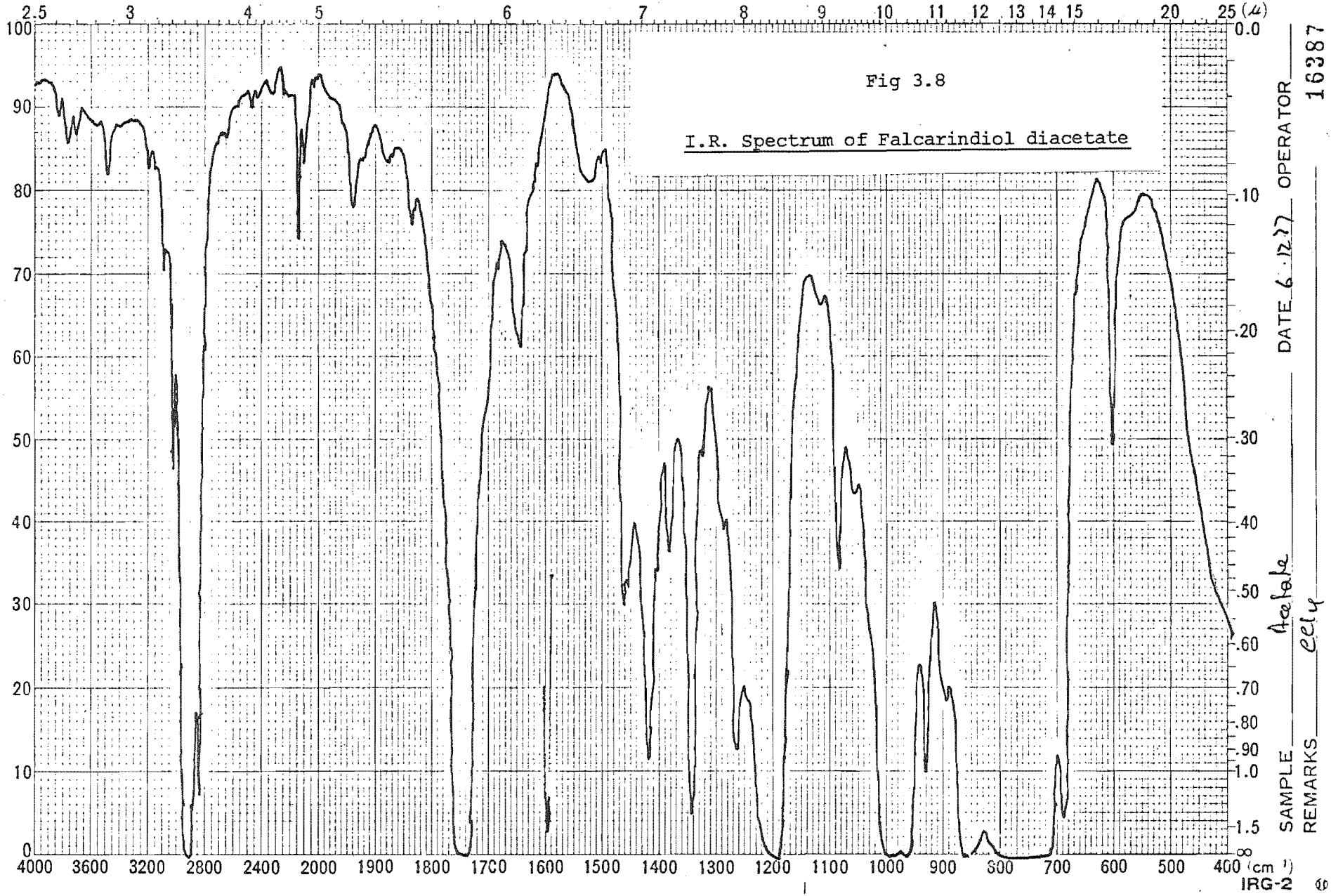


Fig 3.8

I.R. Spectrum of Falcarindiol diacetate



The acetate derivative was subjected to high resolution chemical ionisation mass spectroscopy (HRCIMS) (Fig 3.9) and the accurate masses of the ions produced allowed a formula listing to be produced (see Appendix C). Examination of the data indicated a quasi-molecular ion (M+H) corresponding to a molecular formula of $C_{21}H_{29}O_4$. Also present were ions corresponding to $(M+ NH_4^+)$ and [(M+1)-1]. The ions at m/e 285 (M+H-acetic acid) and m/e 243 ($M+NH_4$-2x acetic acid) indicated a sequential loss of two acetates implying the parent compound must be a diol.

Calculation of the number of double bond equivalents for the molecular formula $C_{21}H_{28}O_4$ gave eight double bond equivalents, (Table 3.13) implying that the molecule is highly unsaturated,

Table 3.13

Calculation of double bond equivalents

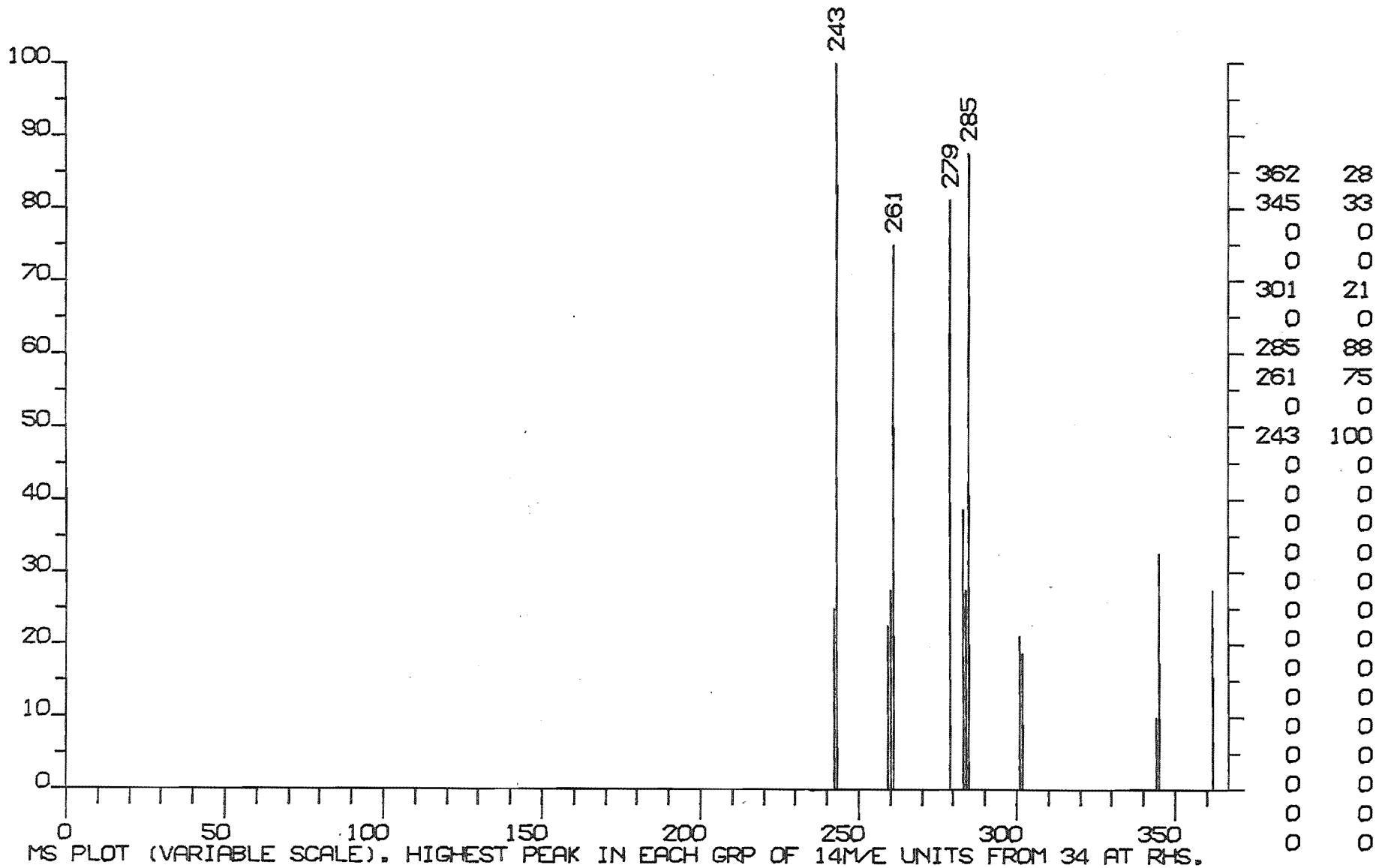
$C_{21}H_{28}O_4$ - (count each oxygen as if a methylene ($-CH_2-$)	
for saturated hydrocarbon	$C_{25}H_{52}$
observe	$C_{25}H_{36}$
	16
Therefore there are 8 double bond equivalents of which two are represented by the acetate functions	

which was confirmed by the I.R. evidence which indicated the presence of both double and triple bonds. The U.V. spectrum of the compound (3.4.14) did not contain the strong absorptions expected if these unsaturated bonds were conjugated, with the exception of a conjugated diyne which does not have an intense U.V. absorption.

The ^{1}H-NMR spectrum of the acetate (Fig 3.10) exhibited two distinct resonances for the methyl protons of the acetate groups (δ_H2.00 and 2.03) (Table 3.14) confirming the presence of a diol in the parent compound. The integral for the methylene proton resonance (δ_H1.26) was calculated by reference to the integral for the methyl triplet (δ_H0.88) and indicated that there were ten protons in an identical chemical environment. This indicated that there was a saturated linear alkyl chain of at least six carbons.

Fig 3.9

HRCIMS of (9Z)-3,8-diacetoxy-heptadeca-1,9-diene-4,6-diyne (M.W. 344)



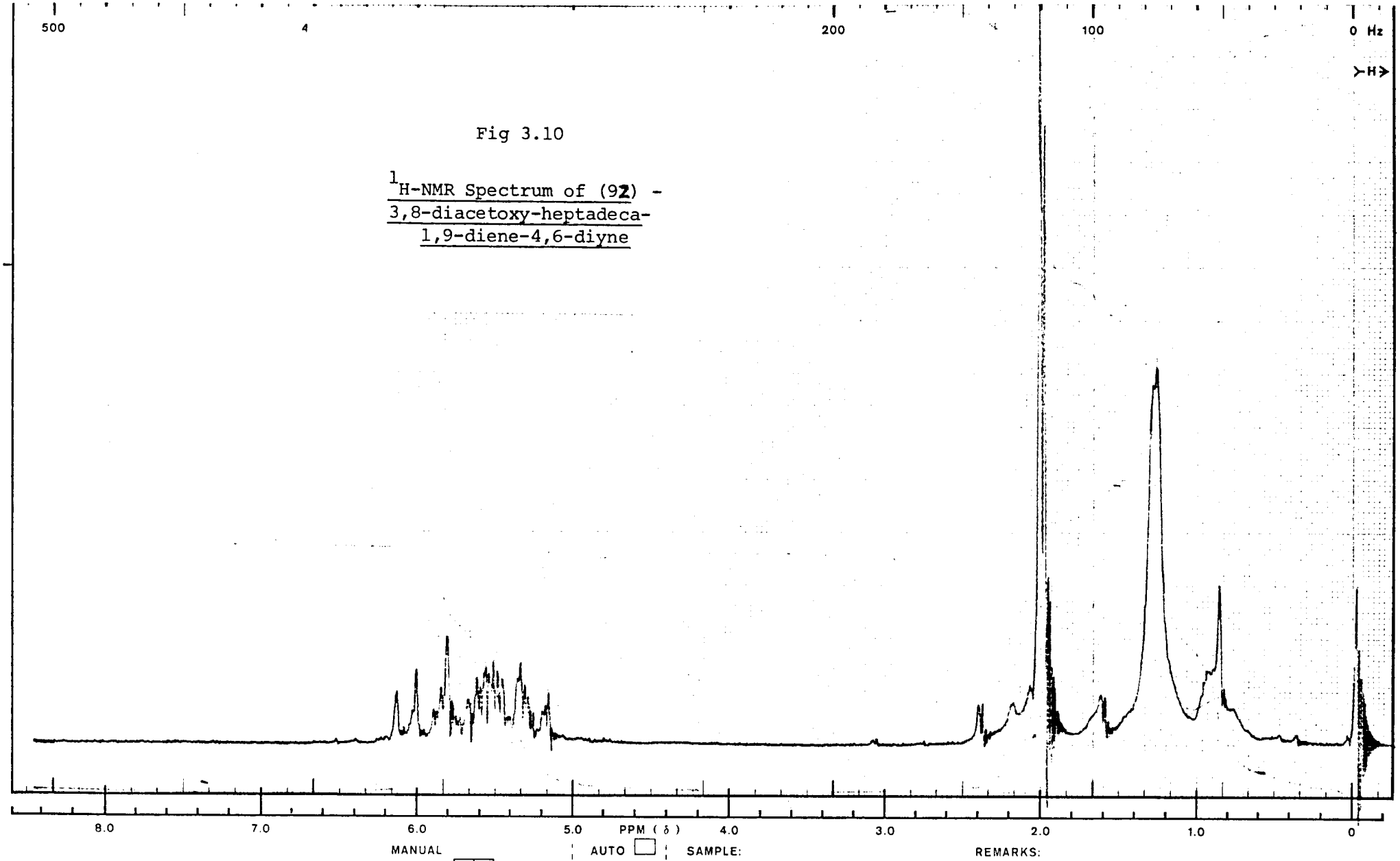


Fig 3.10

^{1}H-NMR Spectrum of (92) - 3,8-diacetoxy-heptadeca-1,9-diene-4,6-diyne

The poorly defined resonances in the region δ_H4.97 to 6.16 integrated for a total of seven protons, the precise assignment of which was not possible. Reference to the published ^{1}H-NMR spectra of C-17 diynes (Table 3.15) indicated that this group of resonances contained two methine protons of the carbons α to the oxygen function, as well as five olefinic protons. Complete assignment of the olefinic resonances has not yet been made but Schulte and Potter (156) were able to assign the resonances at δ_H4.93 and 5.98 to the protons of carbons 2 and 3 of falcarindiol respectively.

Table 3.14

Assignment of the ^{1}H-NMR resonances

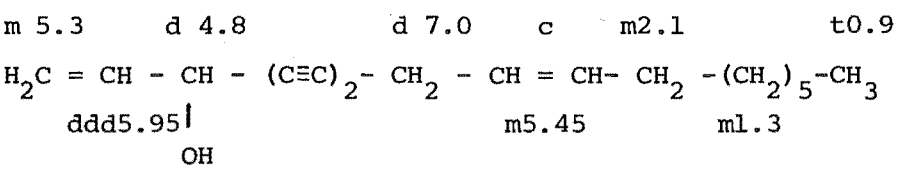
A-Acetate derivative			
δ_H ppm	Multiplicity	Integral	Assignment
0.88	t	3	CH_3-CH_2
1.26	m	10	CH_3-$(CH_2)_5$-CH_2
2.00	s	3	Acetate -CH_3
2.03	s	3	" "
1.6		1	} CH_2-CH_2-CH=
2.2		1	
5.2-6.16		7	-CH=CH-
			CH_2=CH-
			2x (-CH(OAc)-CH=)
B-Diol			
0.90	t	3	CH_3-CH_2-
1.26-1.28	m	10	CH_3-$(CH_2)_r$-CH_2-
2.07	m	2	CH_2-CH_2-CH=
2.97	s	1	-OH
3.6	s	1	-OH
4.97-5.58		7	CH_2=CH-
			-CH=CH-
			2x (C-CH(OH)-CH=)

The chemical shifts of the two methylene protons of the carbon α to the double bond in the acetate derivative appear to be weak and to coincide with the acetate methyl resonances whilst in the diol they resonate as a weak multiplete (δ_H2.07). (Fig 3.11)

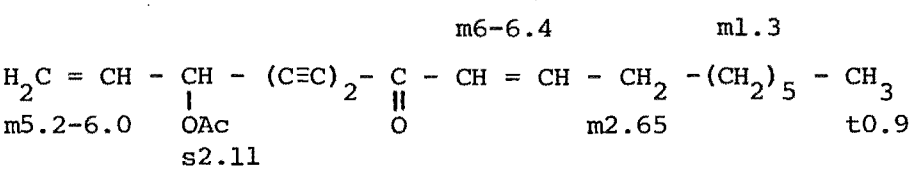
Table 3.15

^{1}H-NMR Chemical shifts of C-17 diynes

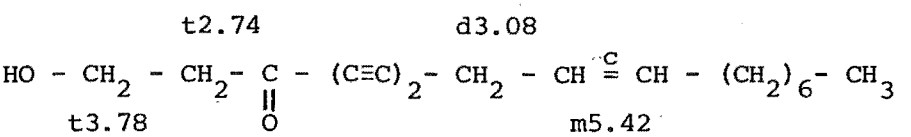
Falcarinol (32)



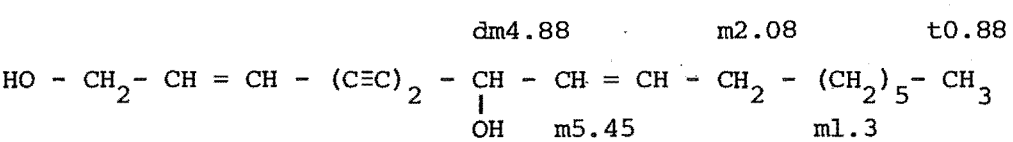
3-acetoxy heptadeca-1,9-diene-4,6-diyn-8-one (30)



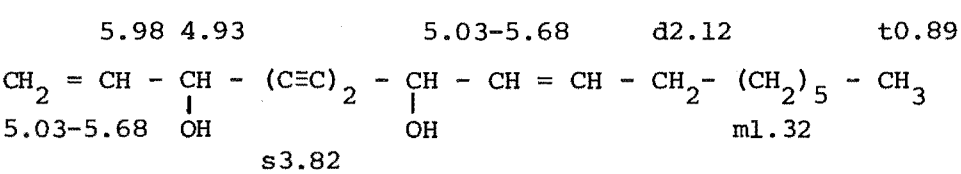
1-hydroxy-heptadaea-9-diene-4,6-diyn-3-one (32)



1-hydroxy-heptadaca-2,9-diene-4,5-diyn-1,8-ol (30)



Falcarindiol (156)



Falcarindiol (15)

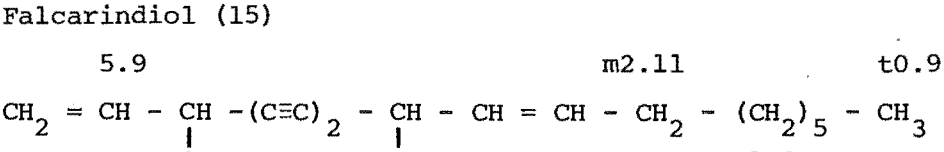
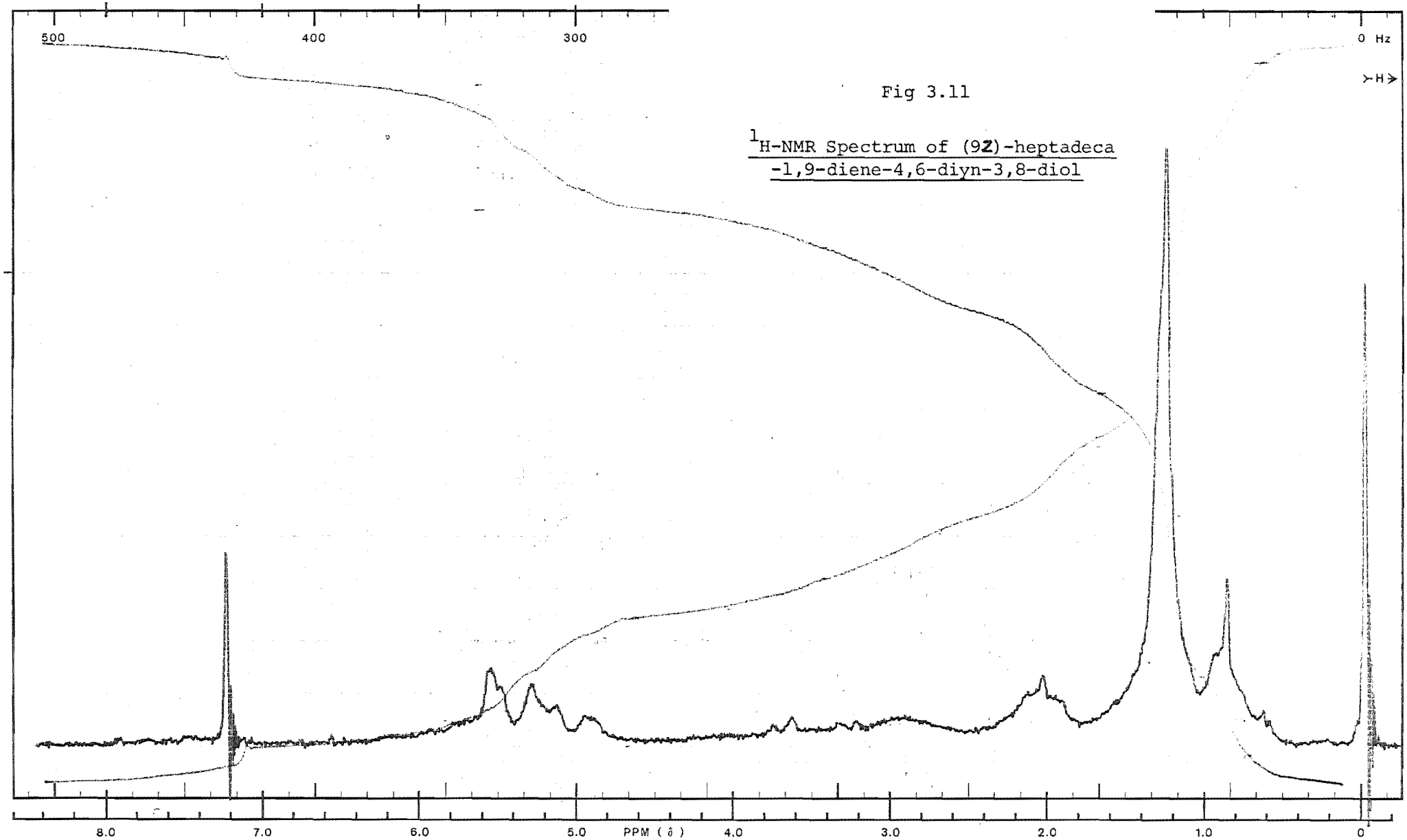


Fig 3.11

^{1}H-NMR Spectrum of (9Z)-heptadeca-1,9-diene-4,6-diyn-3,8-diol



MANUAL | AUTO ☐

SWEEP OFFSET (Hz): _____

SPECTRUM AMPLITUDE: 5.0

INTEGRAL AMPLITUDE: 3.5

SPINNING RATE (RPS): 38

SWEEP TIME (SEC): 50 250 — (250)

SWEEP WIDTH (Hz): 25 50 100 250 500 — (500)

FILTER: 1 2 3 4 5 6 7 8 — (2)

RF POWER LEVEL: 0.027 — (.05)

SAMPLE: Sample C

REMARKS:

SOLVENT: CDCl

The ^{13}C-NMR spectra of the diol (Fig 3.12) and its acetate derivative (Fig 3.13) were obtained as CCl_4 solutions. The carbon count for the diol gave fifteen clearly defined single peaks plus one larger peak due to the coincidence of at least two carbons resonances making a carbon count, in line with the molecular formula implied by HRCIMS of the diacetate, of seventeen. The carbon count for the acetate was only eighteen, due to the coincidence of the acetate resonances and two of the other carbon resonances.

The single frequency offset resonance decoupled spectrum (SFORD) of the acetate derivative allowed the multiplicity of each carbon to be assigned (Table 3.16).

Table 3.16

Assignment of Multiplicity of the Carbon Resonances in the ^{13}C-NMR Spectrum of the Acetate Derivative

Multiplicity[1]	δ_c	Assignment
2q	13.95 20.24	$-CH_3$
3t	22.44 27.54 31.63	$-CH_2-$
1t(large)	28.91	$-CH_2-$
2d	59.19 63.67	-CH-O
4s	69.11 70.60 74.79 76.36	$-(C\equiv C)_2-$
1t	119.04	$H_2C=C$
3d	124.16 132.16 135.53	olefinic
1s	167.49	acetate $\rangle C=O$

1 observed under SFORD conditions

The four resonances in the olefinic region indicated that two double bonds were present. One of these (δ_c119.04), under SFORD conditions resonated as a triplet indicating the presence of two equivalent protons. The carbon which resonated at 119ppm must therefore be the terminal carbon of a vinyl group. The assignment of the δ_c135.53 resonance to the interior carbon of the vinyl group was made by reference to 1-heptene and 2-heptene (A and B Table 3.17). The assignment of the chemical shifts for the other two olefinic carbons was made by reference to compounds B, K & L (Table 3.17).

Fig 3.12

^{13}C-NMR Spectrum of (9Z)
-heptadeca-1,9-diene-4,6-diyn-3,8-diol

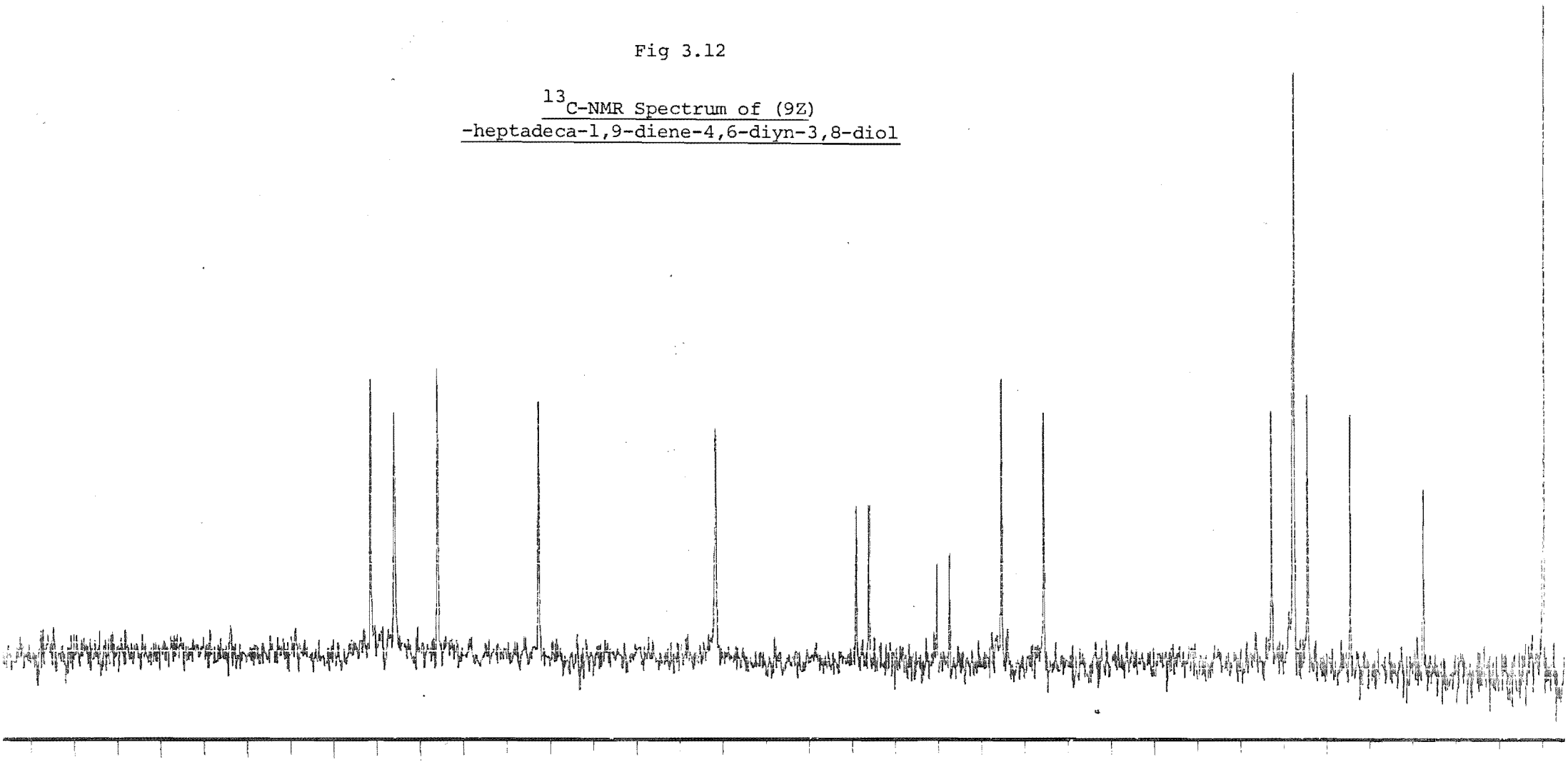
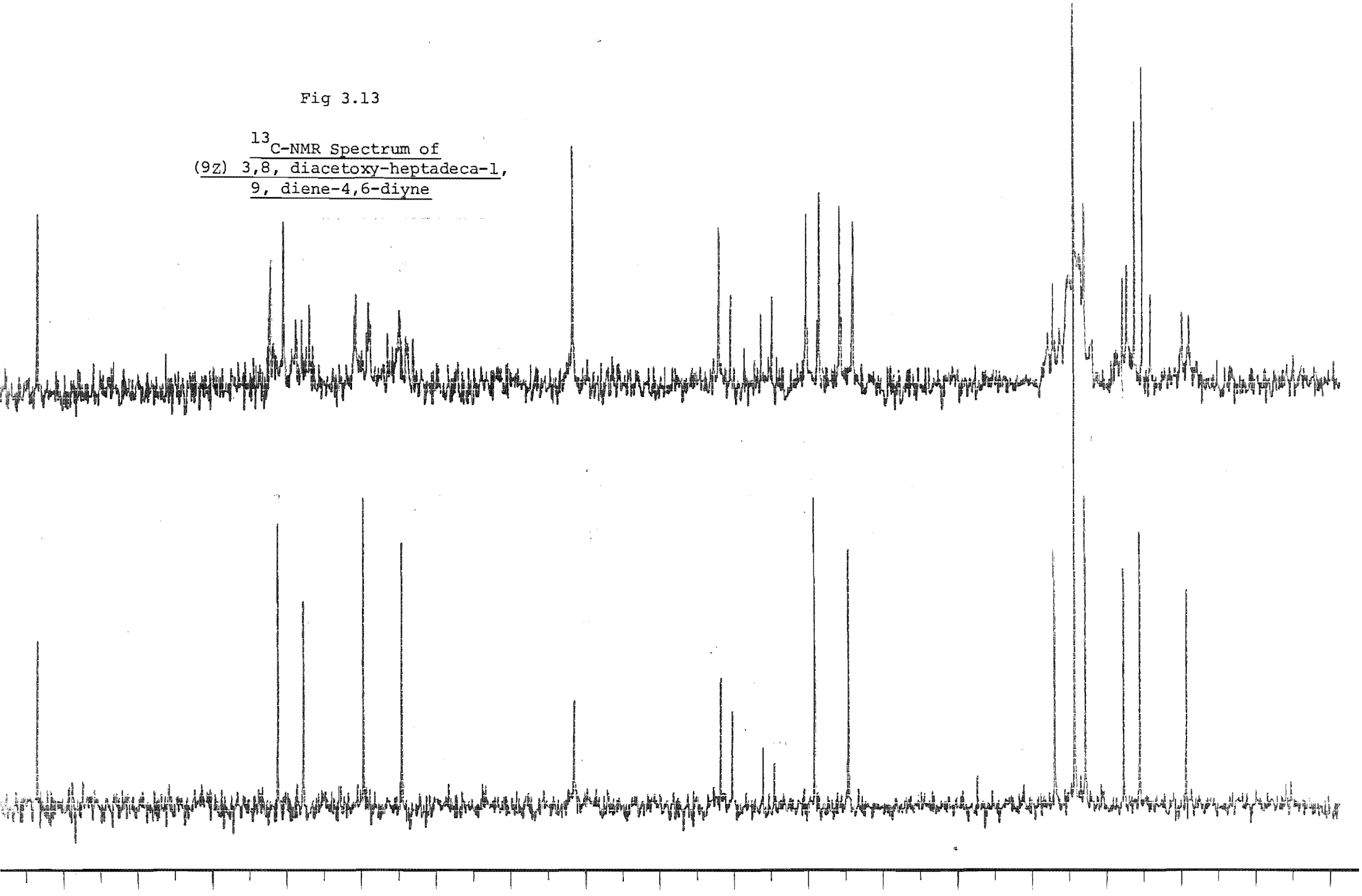


Fig 3.13

^{13}C-NMR Spectrum of
(9Z) 3,8, diacetoxy-heptadeca-1,
9, diene-4,6-diyne



The chemical shifts of the acetylenic carbons in symmetrical mono-acetylenic molecules (Q) are identical, whilst in symmetrical conjugated diacetylenes the inner acetylenic carbons are shielded and resonate 10-12ppm upfield from the outer pair (N) (86). In asymmetric acetylenes (O) the individual acetylenic carbons exhibit different chemical shifts. The presence of two pairs of peaks in the region 63 to 88ppm indicated the presence of an asymmetric conjugated diacetylene. The assignment of the acetylenic carbons (Table 3.16) was made by reference to compounds N. O, & P. (Table 3.17) and are in agreement with the assignments made by Miller *et al* (124) for asymmetric diacetylenes.

The resonances at δ_c 167.49 and 20.24 were assigned to the C=O and $-CH_3$ carbons of the acetate groups respectively (I & J). The δ_c 20.24 resonance was a quartet under SFORD conditions which confirmed the assignment as a methyl group. Resonances at δ_c 63.67 and 59.19 were assigned to the carbons α to the two acetate groups.

With fourteen carbons assigned out of the total of 21 required by the molecular formula, attention was focused on the remaining seven carbons which were inferred (^{1}H-NMR) to occur as a linear chain. The ^{1}H-NMR integral indicated that three was a six carbon alkyl chain. ^{13}C-NMR studies (14, 57) of hydrocarbons have shown that in linear alkanes containing eight or more carbons, coincidence of the resonances of carbons three or more removed from the terminal methyl may occur. If the alkyl chain is attached to other functional groups (C, D, M, R), coincidence of resonances may occur with linear alkyl chains of six or more carbons. The presence of only one unassigned high field quartet (δ_c13.95) indicated that a linear chain was present. A comparison of the observed chemical shifts with those for C-7 alkyl chains indicated a close similarity (Table 3.19).

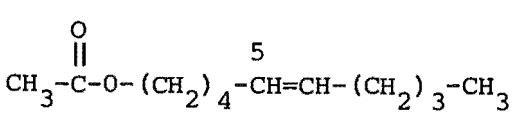
Table 3.17

^{13}C-NMR Chemical Shifts of Model Alkenes

A	$CH_2=CH-(CH_2)_4-CH_3$	1-heptene	(57)
B	$CH_3-CH_2=CH-(CH_2)_3-CH_3$	2-heptene (*cis*)	(57)
C	$CH_2=C(CH_3)-(CH_2)_6-CH_3$		(57)
D	$CH_3-CH=CH-(CH_2)_6-CH_3$		(57)
E	$CH_3-CH_2-CH=CH-CH_2-CH_3$	*trans*	(57)
F	$CH_3-CH_2-CH=CH-CH_2-CH_3$	*cis*	(57)
G	$CH_3-CH=CH-(CH_2)_4-CH_3$	*cis* 2-octene	(100)
H	$CH_3-CH=CH-(CH_2)_4-CH_3$	*trans* -2-octene	(100)
I	$CH_3-CH_2-CH=CH-(CH_2)_3-CH_3$	*cis* 3-octene	(42)
J	$CH_3-CH_2-CH=CH-(CH_2)_3-CH_3$	*trans* 3-octene	(42)

δ_C (ppm) of carbons in position

	1	2	3	4	5	6	7	8	9	10
A	114.20	139.24	34.03	28.96	31.68	22.78	14.06			
B	12.72	123.70	130.98	26.78	32.06	22.58	14.06			
C	109.76	146.04	38.09	27.93	29.51	32.51	32.13	22.89	14.14	
D	17.90	124.59	131.82	32.84	29.45	29.91	29.45	32.14	22.89	14.15
E	14.13	25.94	131.24							
F	14.41	20.70	131.25							
G	12.7	123.6	130.9	27.0	29.5	31.7	22.8	14.1		
H	17.8	124.5	131.7	32.7	29.5	31.7	22.8	14.1		
I	13.3	19.7	130.6	128.4	26.1	20.8	21.6	12.9		
J	12.9	24.9	131.6	128.6	31.6	31.3	21.5	13.0		



(14)

K = *cis* L = *trans*

K	64.8	28.6	26.4	27.1	130.2	131.4	27.3	32.3	22.6	14.1
L	64.8	28.5	26.2	32.5	130.2	131.7	32.6	32.2	22.4	14.1

Table 3.17 (continued)

Model Alkynes

M	$HC\equiv C-(CH_2)_9-CH_3$	(14)
N	$C_6H_5-CH(OH)-C\equiv C-C\equiv C-CH(OH)-C_6H_5$	(86)
O	$C_6H_5-CH(OH)-C\equiv C-C\equiv C-CH_3$	(86)
P	$CH_3-CH_2-C\equiv C-C\equiv C-CH_2-CH_3$	(93)
Q	$CH_3-CH_2-CH_2-C\equiv C-CH_2-CH_2-CH_3$	(100)

	1	2	3	4	5	6	7	8	9	10
M	68.5	85.0	18.6	28.9	30.0	29.2	29.5	30.0	29.7	32.3
N	64.2	79.7	69.9						23.0	14.2
O	64.8	74.2	71.7	63.7	78.2	4.4				
P	13.8	13.2	78.6	65.5						
Q	12.7	21.4	19.2	79.0						

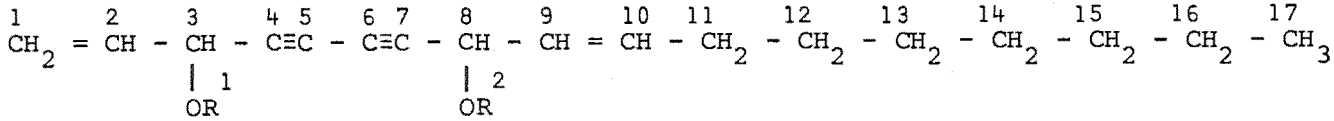
Model Alcohols

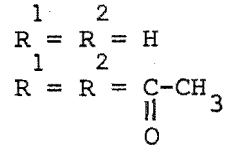
R	$HO-CH_2-(CH_2)_8-CH_3$	1-decanol	(100)
S	$CH_3-CH(OH)-(CH_2)_8-CH_3$	2-decanol	(42)
T	$CH_3-CH_2-CH(OH)-(CH_2)_3-CH_3$	3-heptanol	(148)

R	62.6	32.8	25.9	29.7	29.7	29.7	29.4	32.0	22.7	14.1
S	23.4	67.2	39.6	26.2	30.1	30.0	29.6	32.2	22.9	14.0
T	10.0	29.7	72.6	36.9	28.2					

Table 3.18

Assignment of observed ^{13}C-NMR - chemical shifts of Falcarindiol and its Diacetate derivative





Falcarindiol a

diacetate derivative b

δ_c (ppm) of Carbons in Position

Carbon No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
a.	116.54	135.98	62.84 (1)	79.58 (3)	68.87 (4)	70.30 (4)	78.13 (3)	58.61 (1)	128.24 (2)	133.21 (2)	27.48	29.05	29.22	29.95	31.70	22.51	14.01
b	119.04	135.53	63.67	76.36	69.11	70.60	74.79	59.19	124.16	132.16	27.54	28.91	28.91	28.91	31.63	22.44	13.95

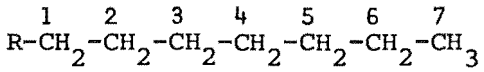
1 - Assignments may be reversed
2 - " " " "
3 & 4

	18	19	20	21
a.	-	-	-	-
b.	167.49	20.24	167.49	20.24

18 & 20 19 & 21

Table 3.19

Assignment of Alkyl Chain Carbons



Observed	I-	Falcarindiol	
	II	Diacetate derivative	
Literature	III	R= $CH_2=C(CH_3)-$	(57)
	IV	R= $CH_3-CH=CH-$ (t)	(57)
	V	R= $CH_3-(CH_2)_2-$	(100)

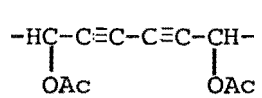
	1	2	3	4	5	6	7
I[1]	27.48	29.05	29.22	29.05	31.70	22.51	14.01
II[1]	27.54	28.91	28.91	28.91	31.63	22.44	13.95
III[2]	38.09	27.93	29.51	29.51	32.13	22.89	14.14
IV[2]	32.84	29.45	29.91	29.45	32.14	22.89	14.15
V	29.9	30.1	30.1	29.8	32.3	22.8	14.0

1 observed in CCl_4 solution

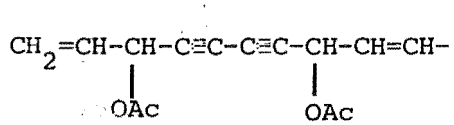
2 observed in $CDCl_3$ solution

NB. Comparison of the δ_c of falcarindiol in CCL_4 and $CDCl_3$ indicated that in CCL_4 the δ_c values were displaced upfield by 0.15 to 0.22ppm.

A number of partial structures emerge from consideration of the spectral evidence. The ^{1}H-NMR and ^{13}C-NMR required a terminal vinyl group, in addition an internal disubstituted double bond was also present along with a conjugated diyne function. ^{1}H and ^{13}C-NMR along with the I.R. established the presence of two secondary acetates and finally an alkyl chain. As neither a conjugated diene or an eneyne system is present it can be assumed that the diyne grouping is flanked by the acetate groupings.

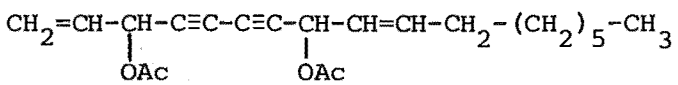


This partial structure can be extended by adding the olefinic bonds one of which must be terminal (*vide supra*)



and the structure can be completed by the addition of the alkyl side chain (Fig 3.14).

Fig 3.14



The configuration of the 9-10 double bond was established as *cis* by comparison with compounds E to J (Table 3.17). In a *cis* configuration the carbon α to the double bond resonates about 5ppm upfield from the equivalent trans isomer, at 27ppm (G & I). The observed chemical shifts for the appropriate methylene carbons in the diacetate and the diol were 27.54 and 27.48ppm respectively.

This established that the active compound was *cis* -heptadeca-1,9-diene-4,6-diyne-3,8-diol a compound previously described by Bentley *et al* (15) and given the trivial name falcarindiol by Bohlmann *et al* (32).

The following spectroscopic data for falcarindiol were given by (i) Bentley *et al* (15): - U.V. (diethyl ether) λ max 258 (ε200), 244 (400) 232(400)nm; I.R. (CS_2) ν max 3600, 3420, 1015 (OH), 984, 933 ($CH=CH_2$), 663 (*cis* -CH=CH-)cm^{-1}; ^{1}H-NMR (CCl_4)τ 9.10 (T,J6Hz,CH_3-), ca8.7br (-$(CH_2)_5$-), 7.89(m,CH_2-CH=), 4.3-5.2 (m,CH_2=CH,CH=CH, and CHOH twice), 4.1 (ddd,J17,9, and 5 Hz,CH_2=CH-CH). (ii) Schulte and Potter (156): U.V. (diethyl ether) λ_{max} 258.7, 244.9, 232.5 nm; I.R. (CCl_4) max 3610, 3350, 1015(OH), 3095, 3025, 1860, 1650, 985, 935 ($CH=CH_2$); 2250, 2145 (C≡C)cm^{-1}; ^{1}H-NMR (CCl_4): δ_H(ppm) 0.89 (t,3H;CH_3), 1.32 (m,$(CH_2)_n$), 2.12 (d,2H;CH_2-C=), 5.03-5.68 (m,5H;4 olefinic & cis =C-CH-C≡), 4.93 (d,J5Hz, 1H) 5.98(m,J5,9.5 and 17Hz,1H) (≡C-CH-CH=CH_2), 3.82 (s,2H,OH).

The following spectroscopic data was given by Bentley *et al* (15) for the diacetate derivative: U.V. (Diethyl ether) λ_{max} 259(E 0.5), 245(1.0) 232(1.0)nm; I.R. (CS_2) ν_{max} 1745, 1215(acetate), 975, 940 (CH_2=CH) 663 (*cis* CH=CH)cm^{-1}; MS. m/e 344(7%), 302(10), 260(100), 259(96).

The assignment of the chemical shifts for carbons 2 to 10 were based entirely on comparison with model compounds, and the effect on the chemical shifts of acetylation of the diol. Acetylation of the -OH results in α downfield shifts, while β carbons shift upfield by an equal amount (42). The observed acetylation shifts for falcarindiol (Table 3.20) exhibited a smaller downfield α acetylation shift than expected and only a small upfield shift for the β vinyl carbon.

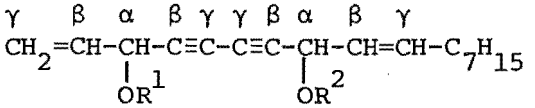
The precise assignment of the resonances of the carbons α to the hydroxyl groups cannot be made since there are no suitable model systems. Examination of the ^{13}C-NMR spectrum of the related compounds (Table 1.7) would provide the necessary information for the complete assignment of the observed chemical shifts.

Similarly the assignment of the acetylenic carbon pairs 4 & 7 and 5 & 6 and the methine carbons 9 & 10 is subject to confirmation by comparison with related compounds.

The assignment of 128.24 and 133.21 to carbons 9 & 10 was made with reference to compounds K, L, I, J, as well as the effect of acetylation. In the model compounds it was noted that the 9 carbon would resonate upfield from the carbon in the 10 position. However examination of the resonance shifts resulting from the acetylation of the diol would suggest that the assignment be reversed. (Table 3.20).

Table 3.20

^{13}C-NMR Acetylation shifts for the acetylation of Falcarindiol



R^1=R^2= H or Acetate

	1	2	3	4	5	6	7	8	9	10
Acetylation shift	+2.5	-0.45	+0.83	-3.22	+0.24	+0.30	-3.34	+0.58	-1.05	-4.08

Expected shifts α +1.5 to 4ppm
β -1.0 to 5ppm

3.4.4 GC-MS of the Diacetate Derivative of Falcarindiol

The diacetate derivative of falcarindiol was subjected to high resolution mass spectroscopy (Fig 3.15) and the accurate masses obtained allowed a formula listing to be produced. (See Appendix C). Although no molecular ion was observed, the fragmentation pattern (Fig 3.16) was consistent with the compound being the diacetate derivative of falcarindiol [1]. The % relative intensities were calculated relative to m/e 141.0693 ($C_{11}H_9$) as 100%.

Fig 3.15

GC-MS of (9Z) 3,8-diacetoxy-heptadeca-1,9-diene-4,6-diyne (M.W. 344).

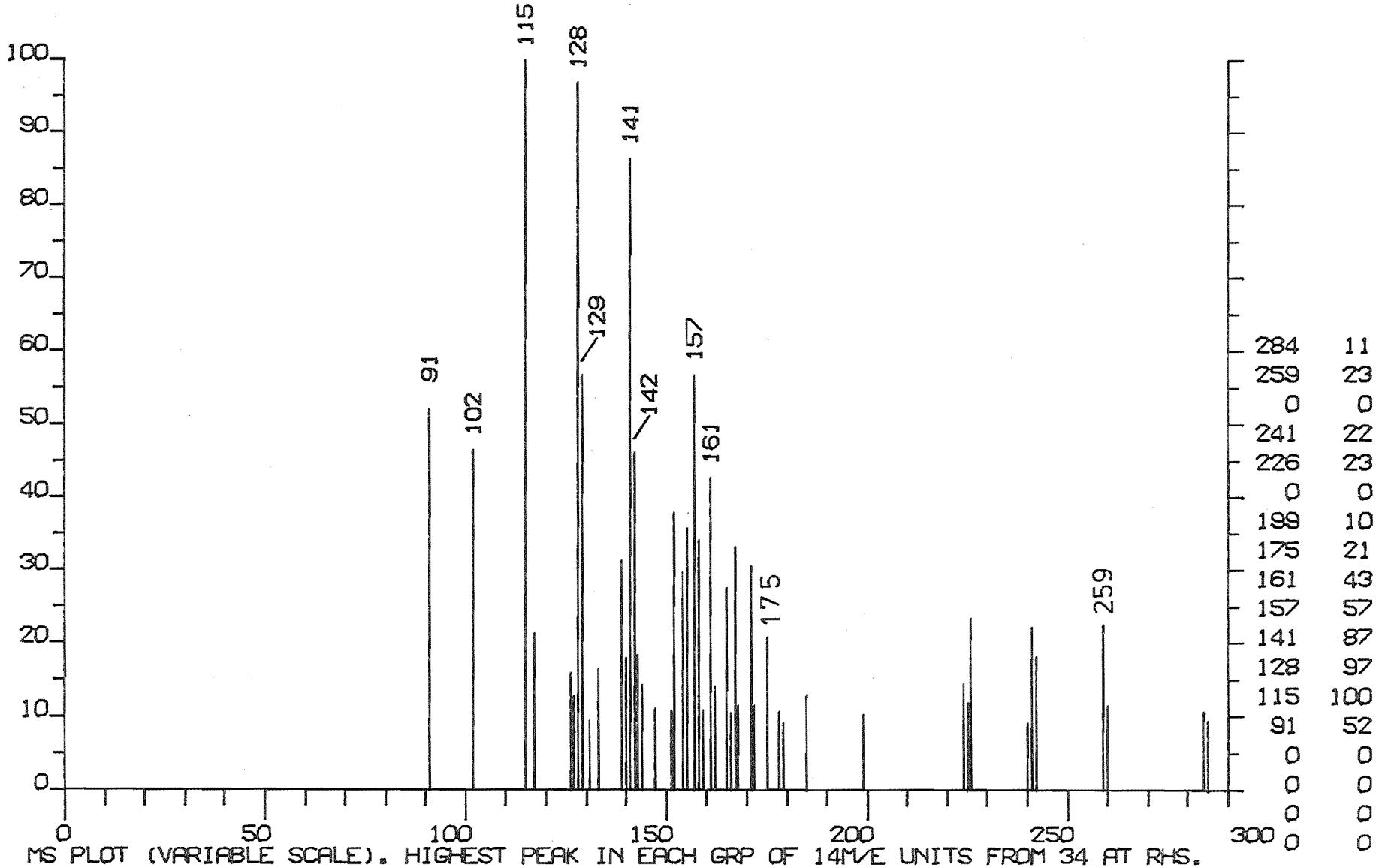
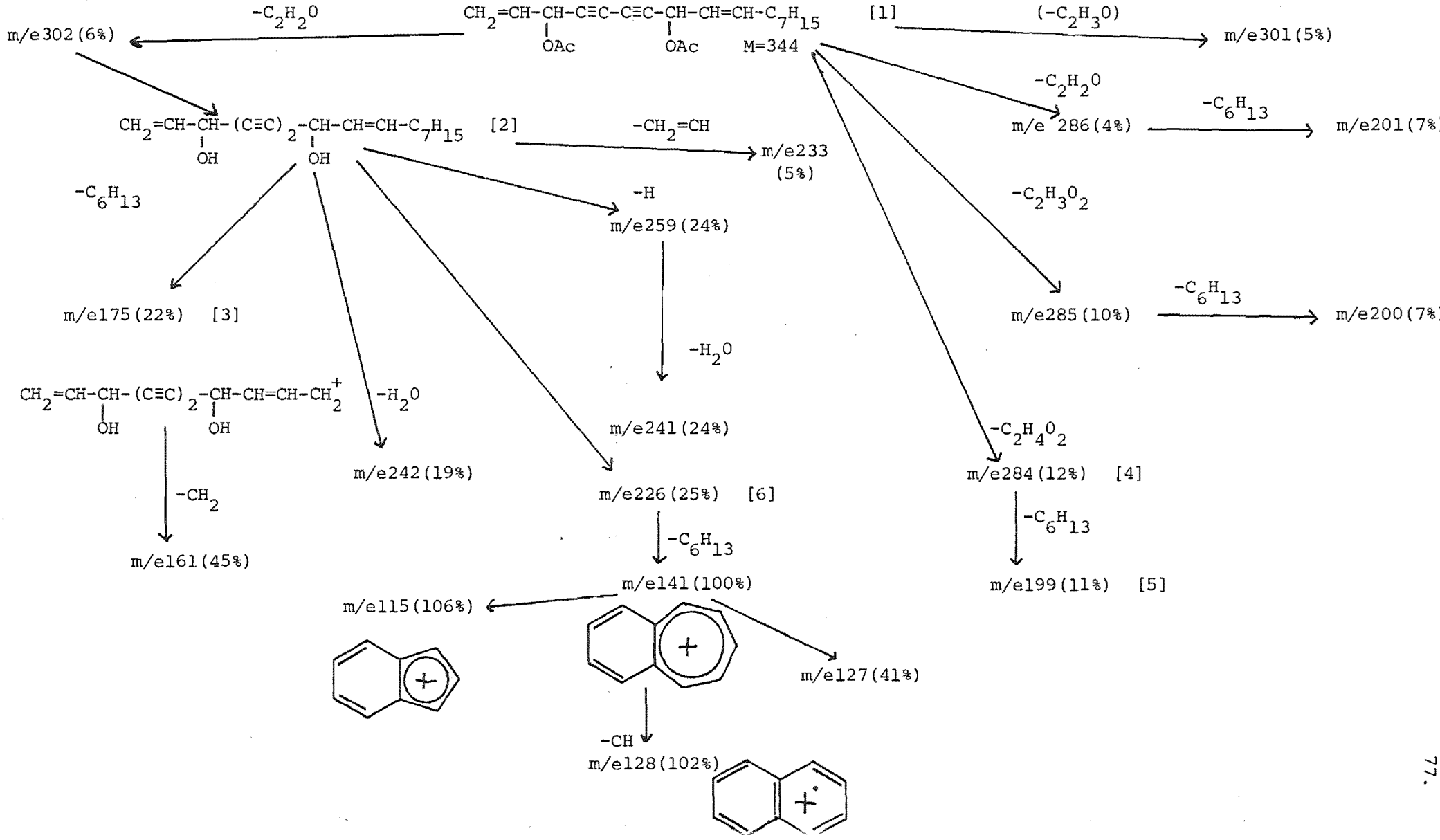


Fig 3.16

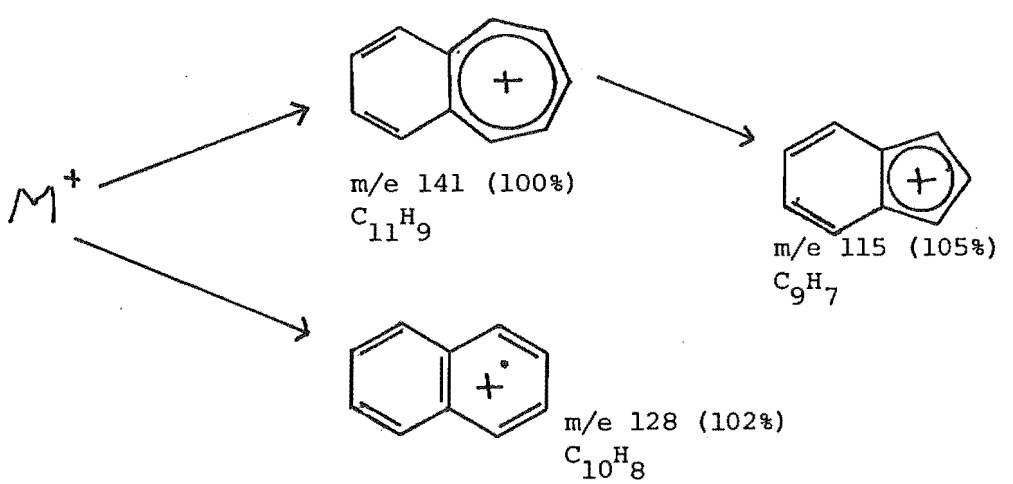
Proposed MS fragmentation of (9Z)-3,8-diacetoxy heptadeca-1,9-diene-4,6-diyne



The 1,9-diene-4,6-diyne unsaturation of C-17 polyacetylenes exhibits a typical allylic fission (30, 101, 144) resulting in the formation of the $M-C_6H_{13}$ [M-85] ion. The observed ion m/e 175 [3] corresponded to the loss of 85 amu from the ion m/e 260 [2] which in turn corresponds to the parent ion in the diol. The ion m/e 199 [5] corresponds to a similar loss of 85 amu from the ion m/e 284, an ion containing one acetate group. Acetate derivatives of long chain alcohols may lose acetic acid ($C_2H_4O_2$) (45). The ion m/e 284 [4] corresponded to the loss of acetic acid from the molecular ion [1] while m/e 226 [6] could have resulted from the loss of two acetic acid molecules.

Schwarz (157) proposed two mechanisms for the elimination of water from α,ω diols such as falcarindiol. The elimination of water may be accompanied by the loss of H or $CH_2=\dot{C}H$, and this mechanism could have given rise to ions m/e 259 and m/e 241. Alternatively water may be eliminated first giving $[M-H_2O]$. The ion m/e 242 could have been formed by elimination of H_2O from m/e 260 [2] or by elimination of $(C_2H_3O)_2O$ from the molecular ion.

The mass spectrum was also characterised by three intense peaks m/e 141, 128 and 115. The accurate masses for these three ions was obtained by high resolution MS and indicated that they were highly unsaturated, with C:H ratios greater than one. These fragments corresponded with the aromatic fragments proposed by Aplin and Safe (9) as common to the fragmentation patterns of a number of polyacetylenes.

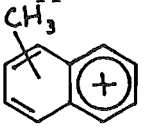


Thus the spectrum contained all the expected ions for the fragmentation of a diacetate of falcarindiol. The published mass spectra for the diacetate (15) lists only 4 fragments m/e 344 (7%), 302 (10), 260(100) and 259(96), of which only the molecular ion m/e 344 was not observed.

3.4.5 Mass spectrum of the TMS derivative

Prior to GC-MS the TMS derivative (2.1.61 and 3.4.3) was chromatographed on a small column of alumina. The column was eluted successively with pentane, 1% diethyl ether in pentane, and methanol. Only the latter fraction contained the compound of interest.

The low resolution (Fig 3.17), and the high resolution mass spectra (Appendix C) revealed the existance of a number of fragment ions typical of polyacetylenes including four intense ions m/e 141, m/e 129



m/e 128 and m/e 115, characteristic of falcarindiol type polyacetylenes (3.4.4). The ion m/e 242 ($C_{17}H_{22}O$) probably represents M-162 [(M- $((CH_3)_3Si)_2$)].

No parent ions were observed, so the configuration of the TMS derivative could not be determined but the high resolution data indicated the presence of a number of Si containing fragments. The TMS group is known to migrate easily as a result of interaction with other functional groups (160), and since falcarindiol is a highly unsaturated compound a large number of re-arrangement ions are possible.

Investigation of the TMS derivative was not pursued further after the identification of the active compound as falcarindiol (3.4.3).

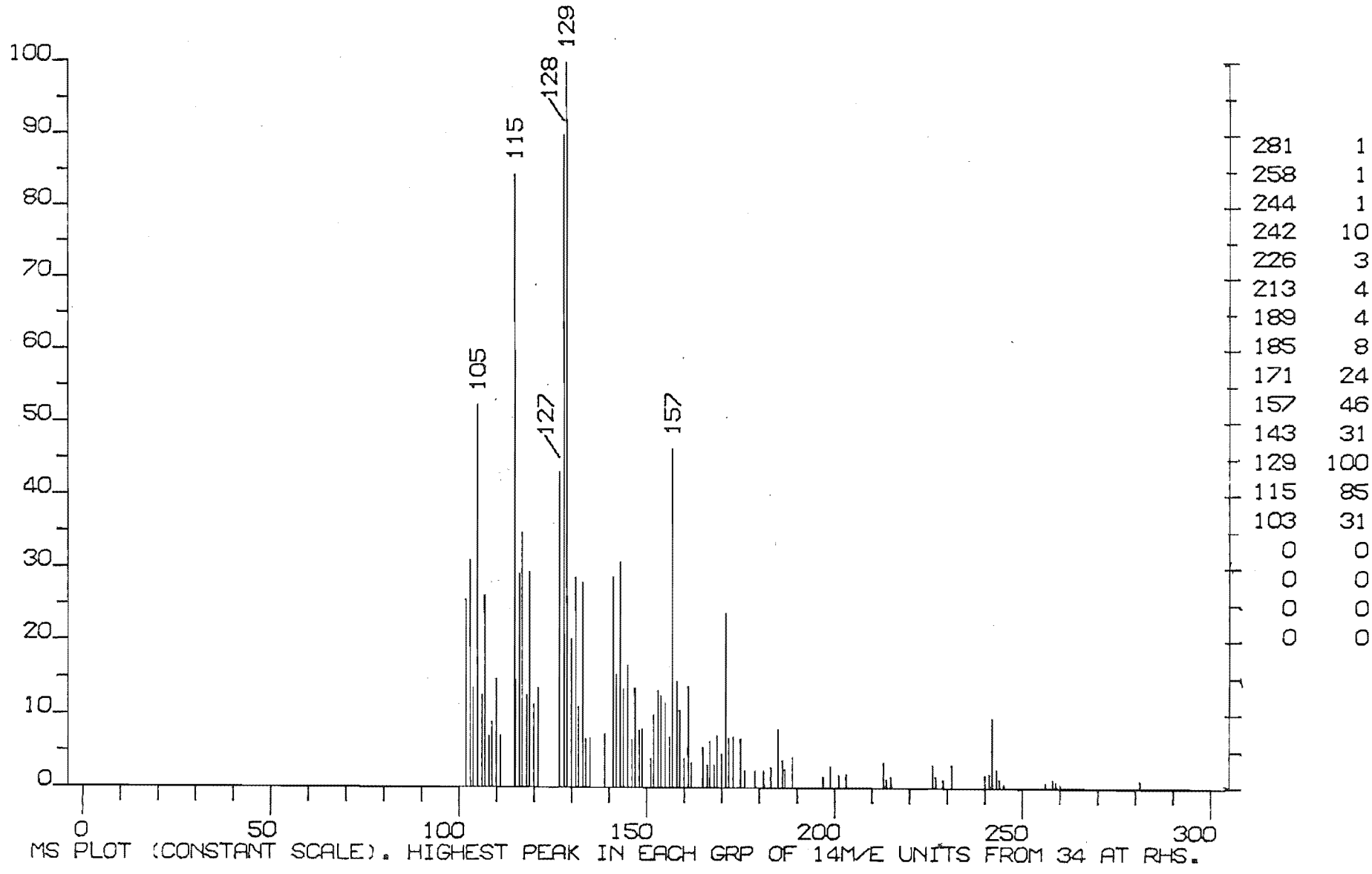
Lifishitz and Mandelbaum (116) recently reviewed the current state of mass spectroscopy of acetylenic compounds.

3.5 Isolation of other compounds from *S. digitata* extracts

3.5.1 Experimental

For details of the instrumentation see 3.4.31. Column chromatography of extracts of *S. digitata* leaves revealed the presence of several unidentified compounds other than falcarindiol. Large quantities of white crystals were obtained from the extracts and identified as *1-hexacosanol* (Ceryl alcohol): I.R. (CCL_4) 3650 3450 (OH), 2960, 2900, 1470(C-H)cm^{-1}; ^{13}C-NMR ($CDCl_3$), 63.13, 32.86, 31.94, 30.17, 30.10, 29.70, 28.99, 25.76, 22.71, 14.12; M.S. ($M-H_2O$) m/e 364.3968. ($C_{26}H_{52}$ requires 364.4069), (M-46) m/e 336.3705 ($C_{24}H_{48}$ requires 336.2756).

Fig 3.17
GC-MS of TMS derivative of Falcarindiol



Purification of falcarindiol resulted in the isolation of a compound with similar chromatographic properties which was identified as the terpene Phytol: M.S. (M^+) m/e 296.3103 ($C_{20}H_{40}O$ requires 296.3079), ($m-H_2O$) m/e 278.2991 ($C_{20}H_{38}$ requires 278.2964), m/e 123.1131 (C_9H_{15} requires 123.1170), m/e 71.048 (C_4H_7O requires 71.0495) m/e 71.086 (C_5H_{11} requires 71.0858). Preparation of the acetate derivative gave (M) m/e 338.3107 ($C_{22}H_{42}O_2$ requires 338.3185).

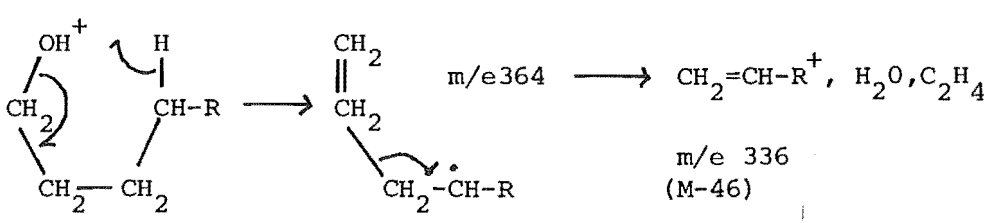
3.5.2 Identification of Ceryl Alcohol (1-hexacosanol)

After concentration fraction F from the alumina column (3.4.22) yielded a white crystalline material which was soluble in diethyl ether but largely insoluble in hexane. (0.5% of D.Wt). These crystals were recovered by filtration and recrystallised from warm hexane and then chromatographed on Silica gel TLC in ether, petroleum ether (3:7). No zones were observed when the TLC plates were sprayed with isatin or phosphomolybdic acid, however crystals were recovered from the zone R_f0.20-0.30. The I.R. spectrum was obtained in CCl_4 and revealed weak OH absorptions plus strong C-H stretching and deformation absorptions. (Fig 3.18). The sample was examined by ^{13}C-NMR (Fig 3.19) which revealed chemical shifts which confirmed the presence of a hydroxyl group and a long saturated alkyl hydrocarbon chain.

High resolution MS gave fragments m/e 364 and m/e 336 consistent with the loss of H_2O and $CH_2{=}CH_2^+$ from a long chain alcohol (see Fig 3.20). Examination of the rest of the fragmentation pattern (Fig 3.21) revealed an even cascade of 14 amu (CH_2) characteristic of saturated linear hydrocarbons. The fragments observed are consistent with the expected fragmentation of an alcohol with the molecular weight of 382 ($C_{26}H_{54}O$).

Fig 3.20

Fragmentation of Ceryl Alcohol



M=382

(Budzikiewicz) (45)

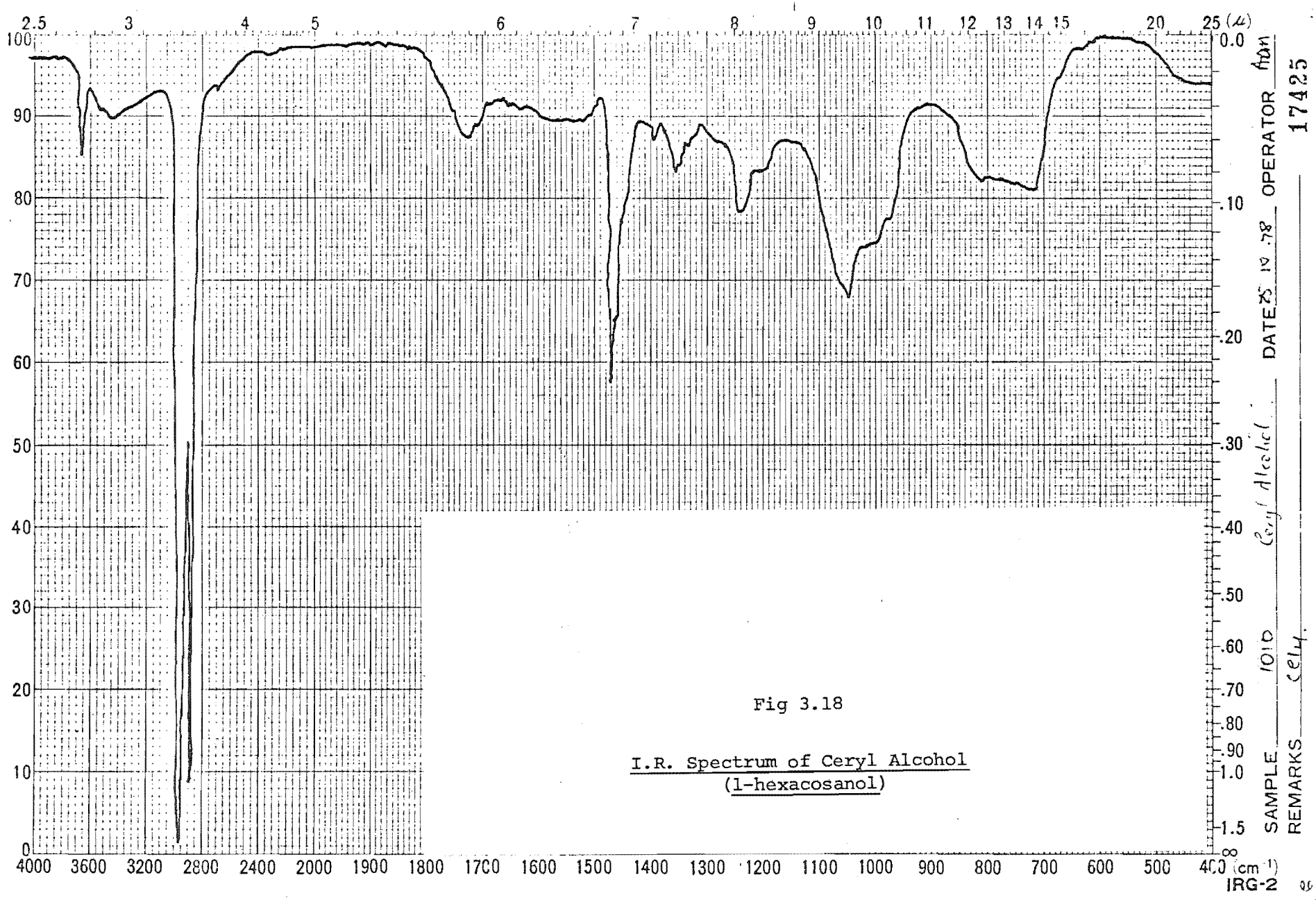


Fig 3.18

I.R. Spectrum of Ceryl Alcohol
(1-hexacosanol)

Fig 3.19

^{13}C-NMR Spectrum of
1-hexaneosanol (Ceryl Alcohol)

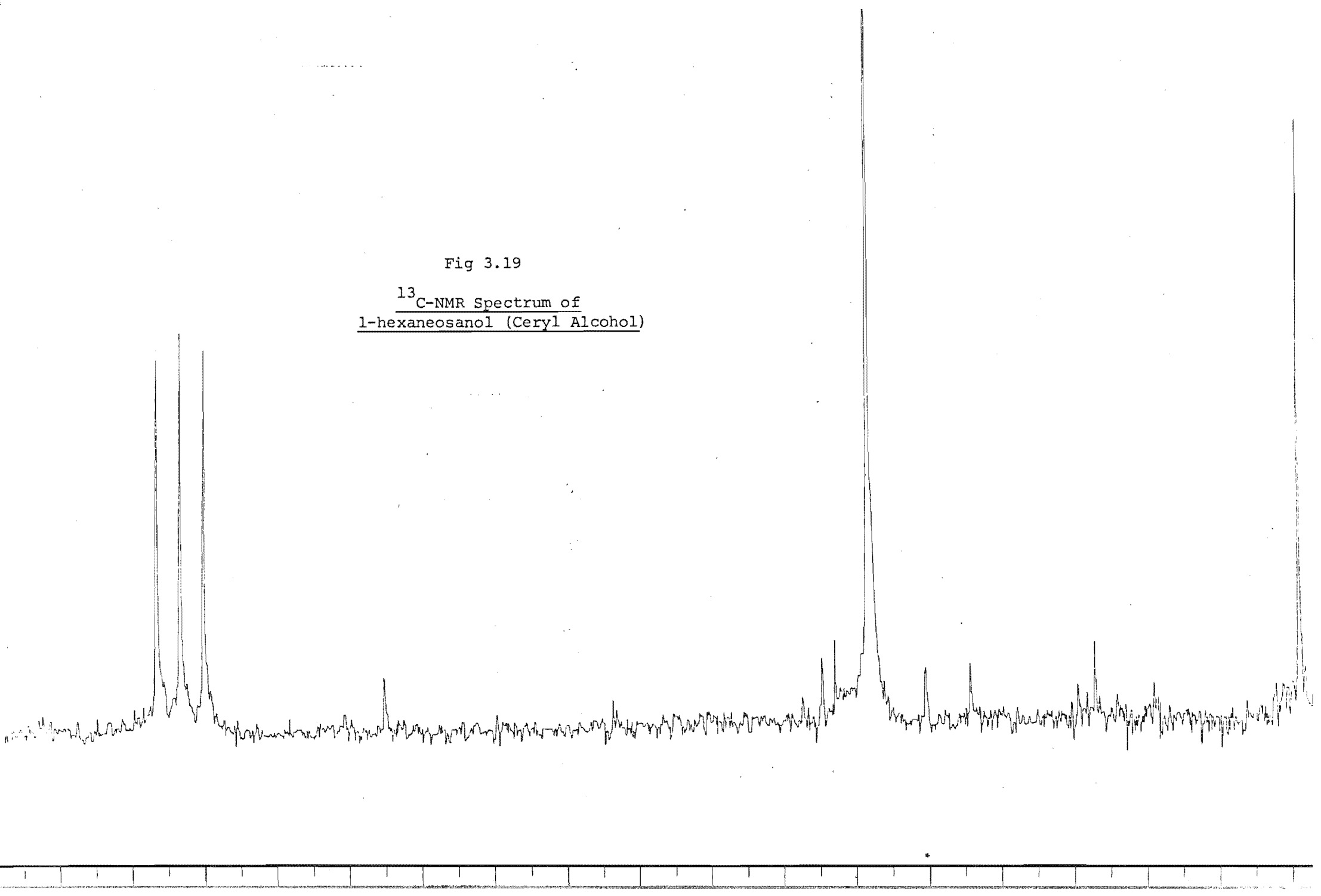
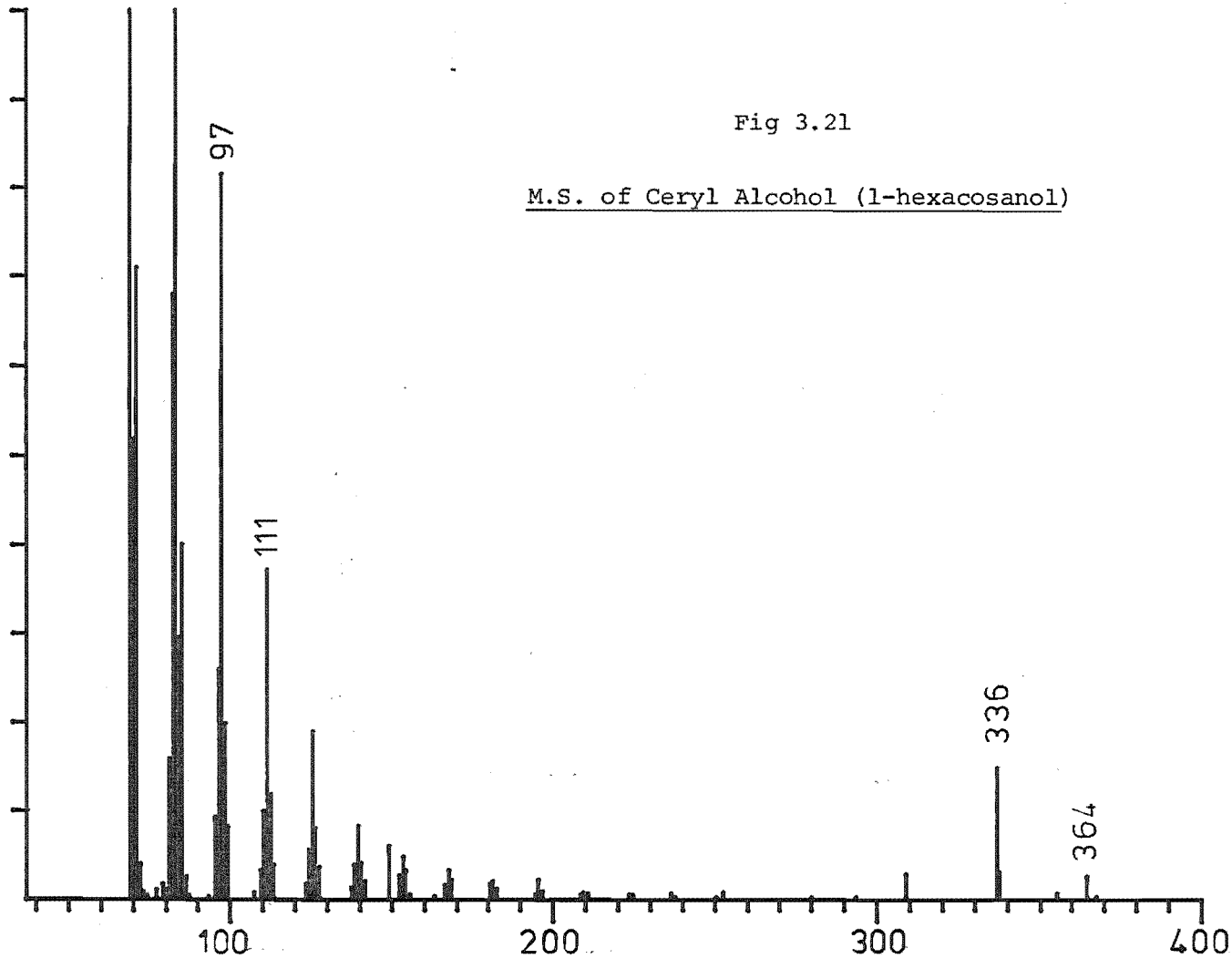


Fig 3.21

M.S. of Ceryl Alcohol (1-hexacosanol)



Assignment of the ^{13}C-NMR chemical shifts were made by reference to the known δ_c of n-decanol (see Table 3.21). The amalgamation of resonances at 29ppm is an established feature of long saturated hydrocarbon chains. (See 3.4.32).

Table 3.21

Assignment of Chemical Shifts of Ceryl Alcohol

$$CH_3^{26}-CH_2^{25}-(CH_2)_{22}-CH_2^{2}-CH_2^{1}-OH$$

(ppm) of carbons in position

	1	2	3	4	5	6-22	23	24	25	26
I	63.13	32.86	25.76	30.10	30.17	29.70	28.99	31.94	22.71	14.12
II	62.20	33.2	26.4	30.1	30.1	30.2^{6}	29.9^{7}	32.5^{8}	23.1^{9}	14.3^{10}

I observed spectrum of Ceryl Alcohol (in $CDCl_3$)
II -n-decanol (100).

3.5.3 Isolation and Identification of Phytol from *S. digitata* Leaves

Fractions eluting from the alumina column (3.4.22) before falcarindiol were pooled and rechromatographed on 10% deactivated alumina. The fractions eluting between 11 and 14 bed volumes were combined and chromatographed on Silica gel PF $_{254+366}$. A zone (R_f 0.19-0.29; diethyl ether,petroleum ether (1:4)) which appeared blue under U.V., was recovered and rechromatographed on silica gel GF_{254}. A yellow-brown spot was observed when the TLC plates were sprayed with phosphomolybdic acid (2.1.442) and heated.

High resolution mass spectroscopy gave a molecular ion m/e 296. ($C_{20}H_{40}O$). The fragmentation pattern is in agreement with that observed for phytol (117). The characteristic ions were m/e 278 ($M-H_2O$), m/e 123 and the double peaks of m/e 71.048 ($HO-CH_2-CH=C-CH_3$) and 71.086 ($C_5H_{11}^+$). Preparation of the acetate derivative gave a compound with a molecular ion of 338. ($C_{22}H_{42}O_2$). An intense peak at m/e 278 (M-acetic acid), characteristic of acetate derivatives was present. The rest of the fragmentation pattern was similar to that recorded for phytol with intense ions m/e 123, 71.0869 and 71.0513.

The sample was found to be impure when subjected to NMR spectroscopy with traces of a long chain linear alcohol present.

Insufficient material was available for further analysis.

4.0 MODE OF ACTION OF FALCARINDIOL

A number of simple experiements were devised to investigate the *in vitro* action of falcarindiol as it affected the macroconidia of *M. gypseum*.

4.1 Optical Microscopy

Examination of *M. gypseum* macroconidia treated with falcarindiol indicated that dramatic changes had occurred. Untreated spores exhibited normal spore structure (Plate 4.1A & B), while those treated with falcarindiol exhibited a severely disrupted cytoplasm (Plate 4.1 C & D). Here the plasma membrane appeared to have shrunk away from the cell wall with a corresponding increase in optical density, making these spores easily recognised. A large number of treated spores were examined and none of them were seen to germinate after being observed in this condition.

Macroconidia of *M. gypseum* treated with falcarindiol diacetate (3.4.3) were also inhibited but did not show the characteristic internal shrinkage; rather they tended to swell and a few were observed to rupture.

In order to test the effect of falcarindiol of growing mycelium, spores were allowed to germinate for 24 hours prior to exposure to falcarindiol (Plate 4.2). Within one hour of exposure the germinated spores appeared to exhibit many of the features of the ungerminated spore after exposure to falcarindiol. Observation of the contents of the hyphal elements themselves was not possible under the optical microscope without fixing the material.

4.2 Transmission Electron Microscopy

Observation of treated and untreated *M. gypseum* spores in the electron microscope confirmed and extended the optical microscopic observations.

The untreated spores exhibited characteristic fungal structures with the presence of many membrane bounded vesicles and very pronounced protrusions on the exterior of the cell wall (Plate 4.3). By contrast the treated spores (Plate 4.4) showed a distinct lack of cellular organisation with a markedly granular appearance and irregular shaped electron dense areas within the spore lumen.

Plate 4.1

Optical Microscopy of *M. gypseum* macro-conidia. A (Phase contrast) Germinating spores. (control). (170X). B. (Direct Lighting) as for A (120X). C. (Phase contrast) Spores treated with 40 µg/ml Falcarindiol (170X). D. (Direct lighting) as for C (90X).

Plate 4.2

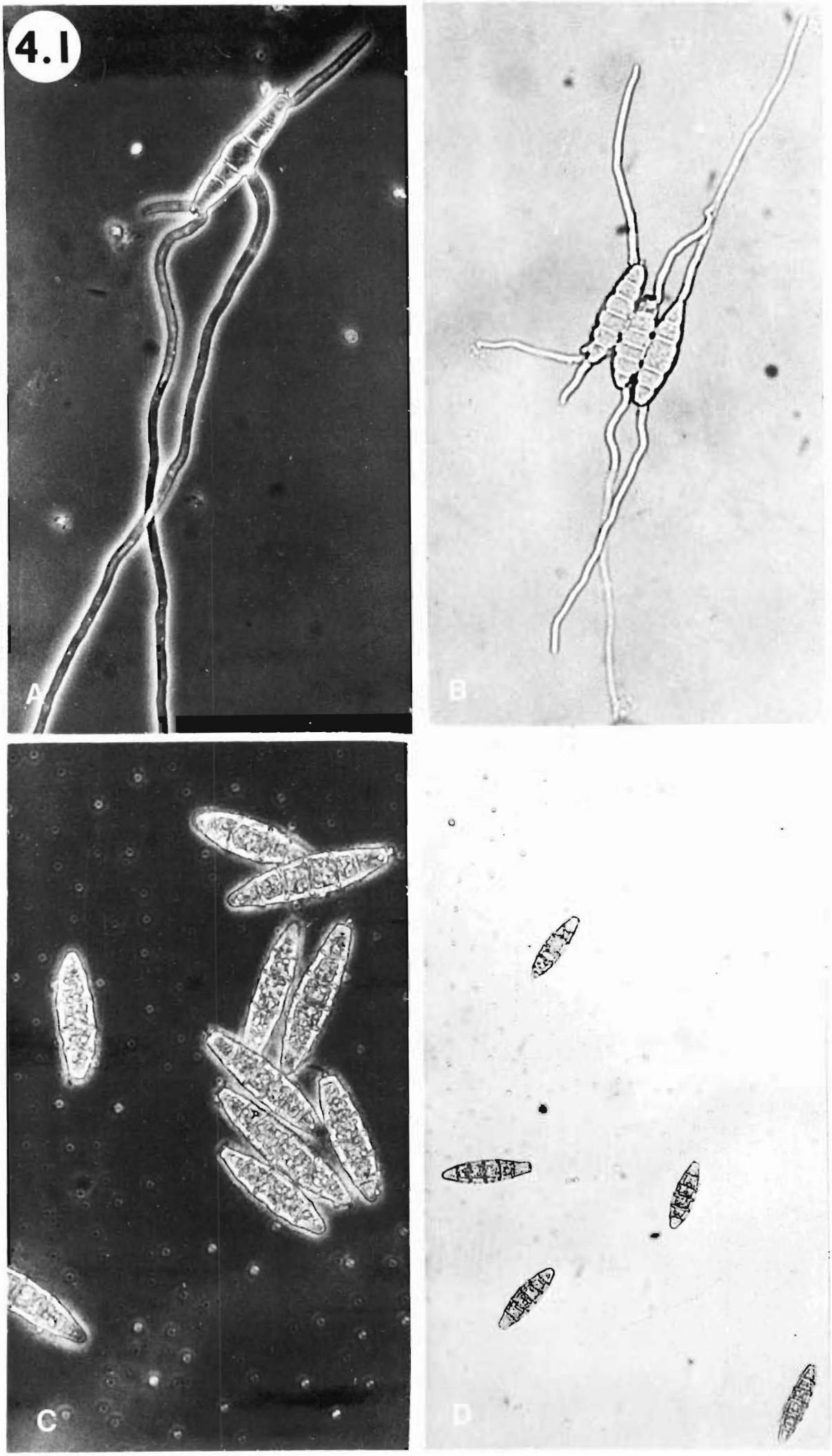
Germinated Spores of *M. gypseum* treated with Falcarindiol. (phase contrast). A. Control (180X). B. 2 hours after application of 40 µg/ml of Falcarindiol (190X).

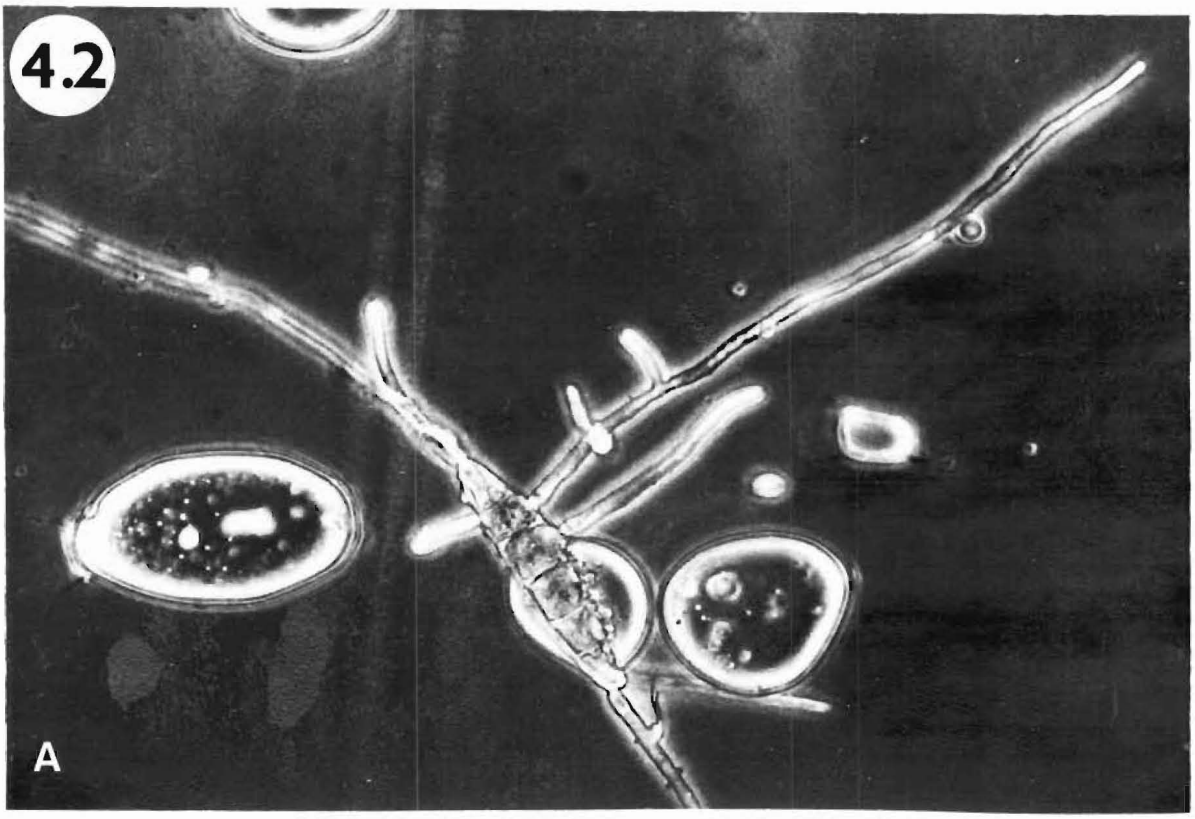
Plate 4.3

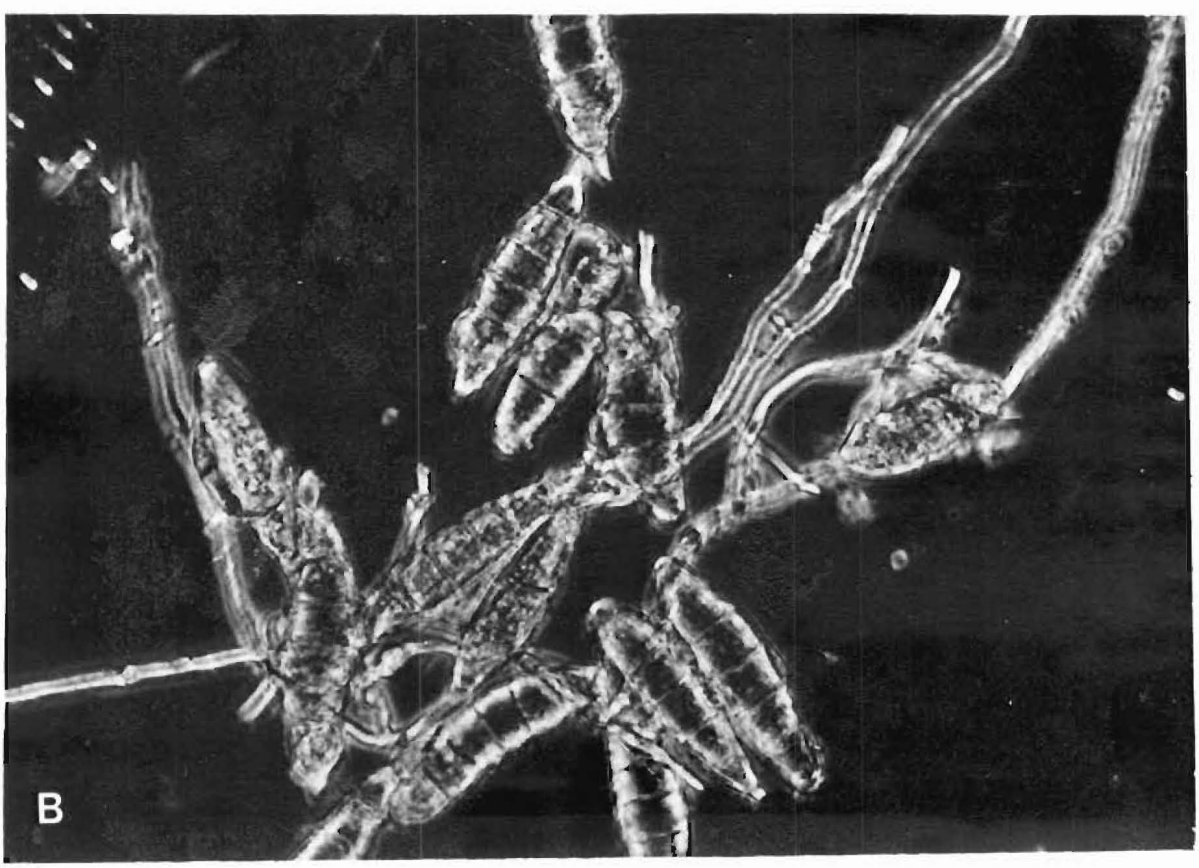
TEM of *M. gypseum* spores. Control. A. (5900X), B. (7000X), C. (8000x), D. (1700X).

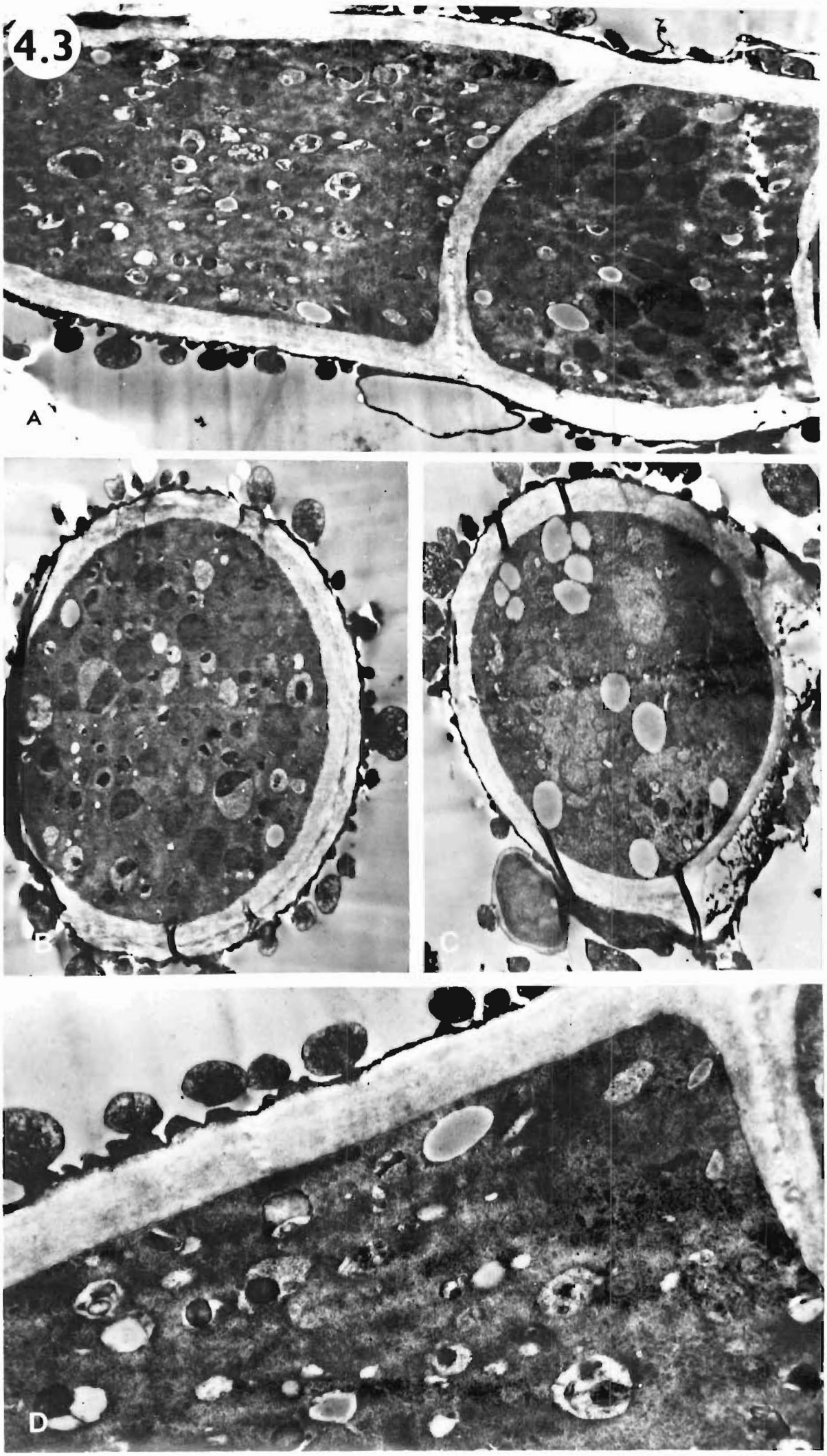
Plate 4.4

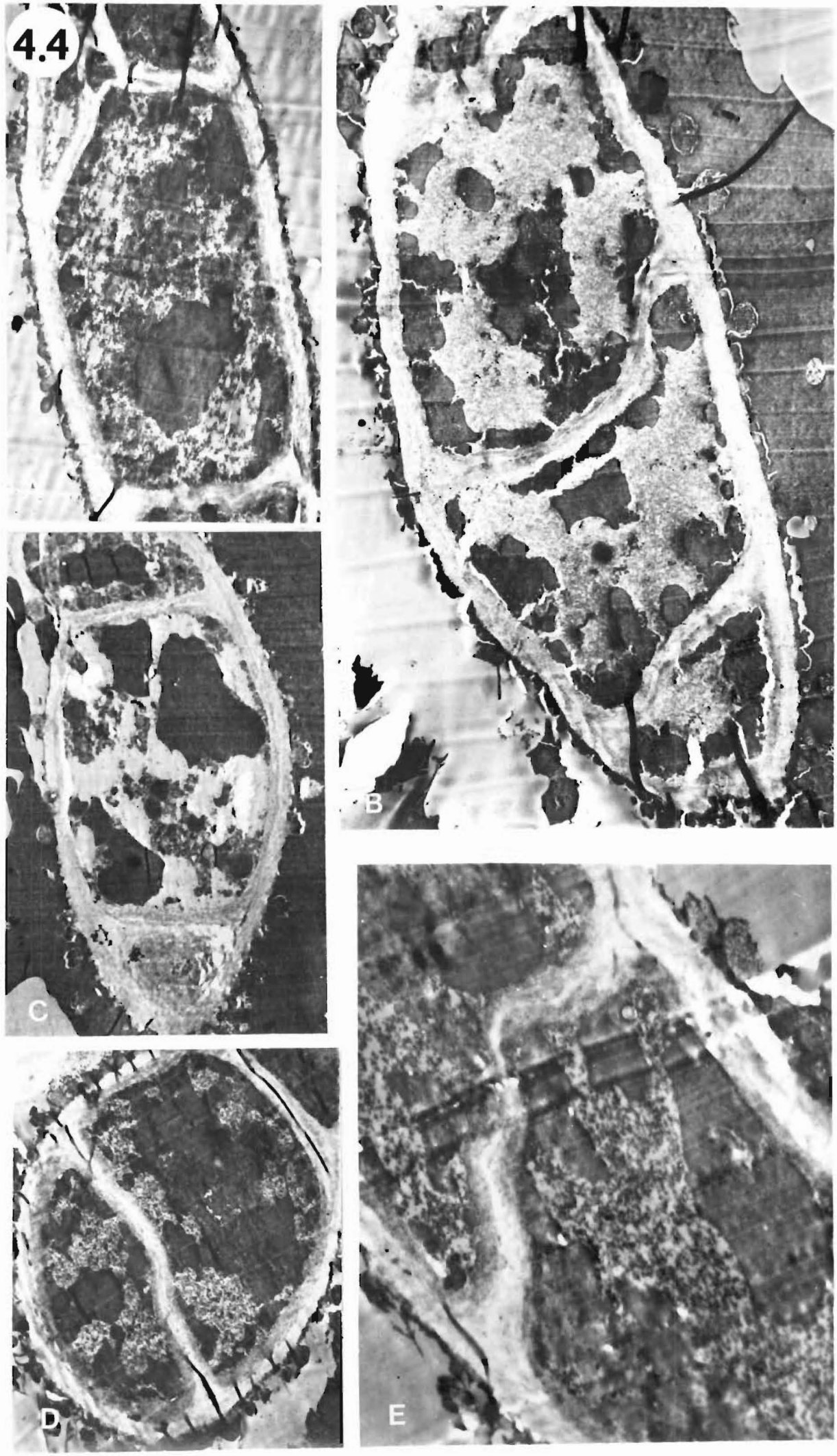
TEM of *M. gypseum* spores treated with 40 µg/ml Falcarindiol. A. (5600X), B. (5900X), C. (4300X), D. (6400X), E. (11400X).


4.1
A
B
C
D


4.2
A


B


4.3
A
B
C
D


4.4
B
C
D
E

This effect was quite unlike anything which has been observed by other workers when investigating the effects of other antifungal agents on dermatophyte spores (25, 134, 176). Spores in this condition are unlikely to be viable. Examination of the specimens at higher magnifications indicated a significant change in the thickness of the cell wall indicating that the primary effect of falcarindiol may be on the wall itself. (Plate 4.5).

4.3 Effect of Falcarindiol on *M. gypseum* grown in Shaken Liquid Culture

This experiment was designed to test the effect of falcarindiol on the mycelial phase of dermatophyte growth since it was recognised that in most clinical situations the mycelium would be ramifying in the affected tissues. The cultures were agitated to prevent the formation of a surface myselial mat.

Two ml of a spore suspension of *M. gypseum* in Sabouraud broth was added to sterile test tubes which were then incubated on an orbital shaker in an incubator at 35°C. Falcarindiol was added to the cultures in 50 µl aliquots of diethyl ether at the following concentrations - 0,50 and 100 µl/ml, after incubation periods of 24, 48 and 96 hours. The cultures were incubated for a further 48 hours and the effect measured. (See Table 4.1). (Plate 4.6).

The cultures were removed from the shaker and observed for a

Table 4.1

The effect of Falcarindiol on the Mycelial growth of *M. gypseum*

Falcarindiol µg/ml	Pre-incubation period (Hours) 24	48	96
0	+	+	+
50	-	-	-
100	-	-	-

(+ = visible mycelial growth subsequent to treatment with Falcarindiol.
- = no further mycelial growth.) (Triplicate)

further two weeks, at which time some growth began on the 96 hour pre-incubation cultures. Subsequent agitation of these cultures suppressed growth for a period of time. These results confirmed the

Plate 4.5

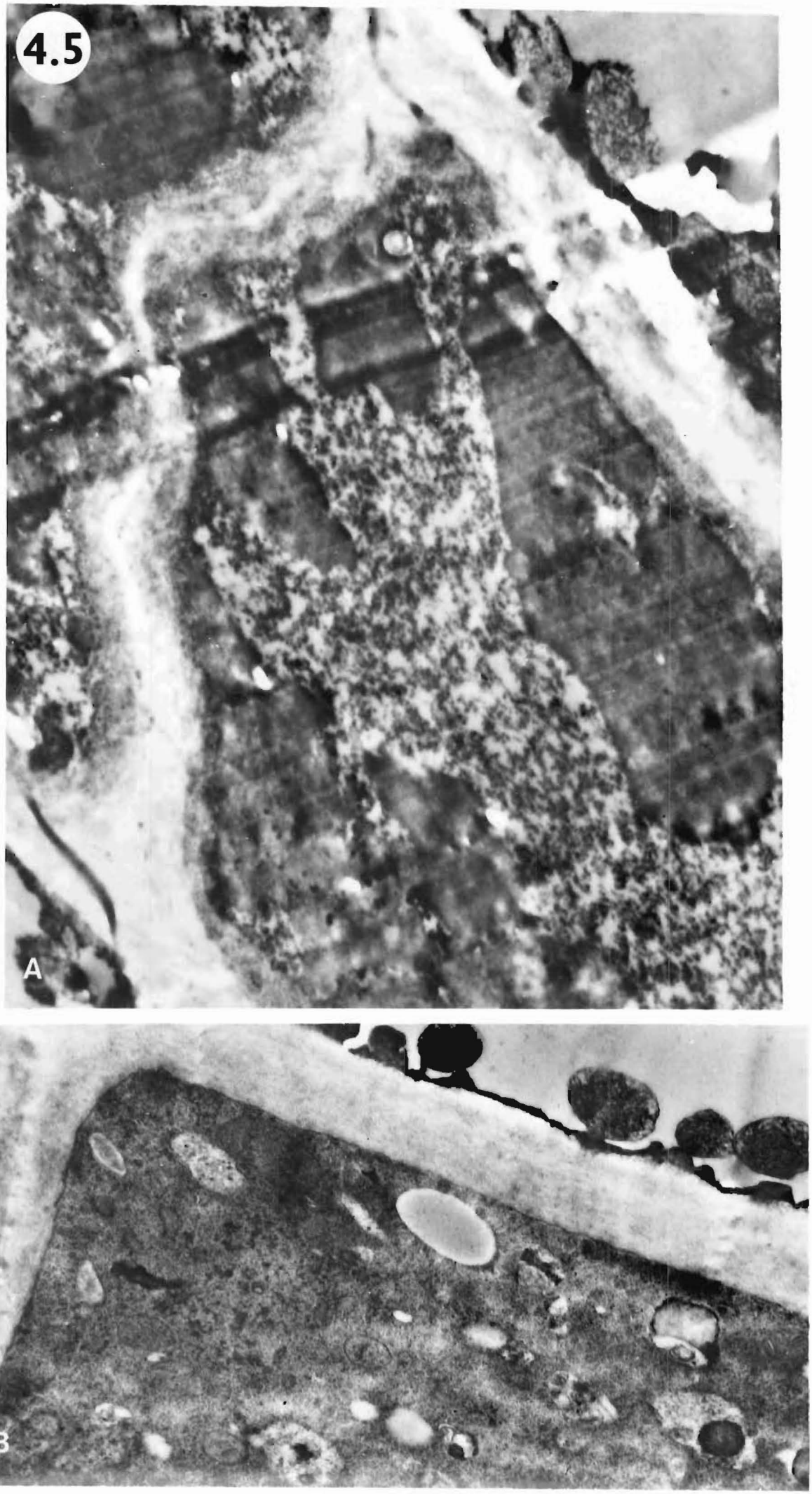
TEM of *M. gypseum* spore walls.

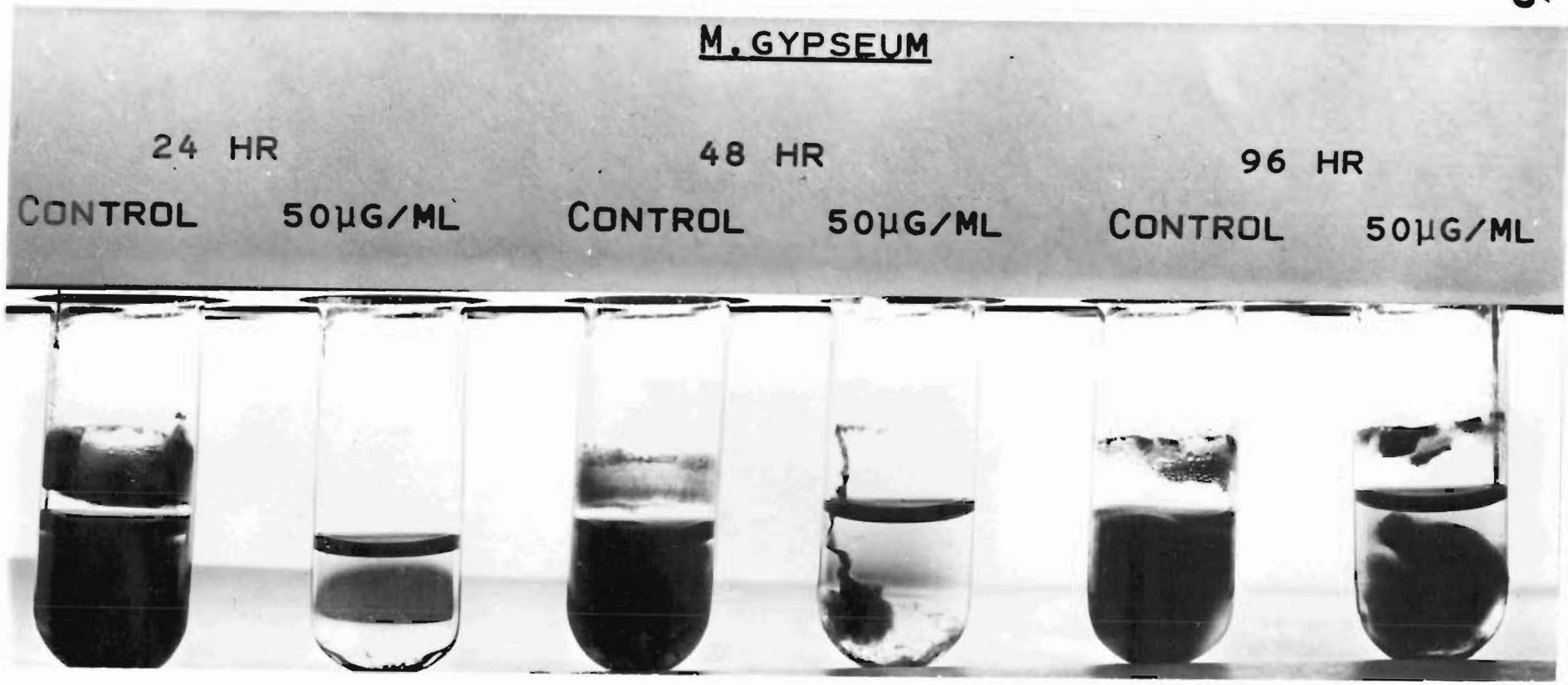
A. Spores treated with 40 µg/ml Falcarindiol (22100X).

B. Control spores (22100X).

Plate 4.6

Effect of Falcarindiol on *M. gypseum* grown in liquid culture. Observed after 4 days incubation on orbital shakes.


4.5
A
B


M. GYPSEUM
24 HR
CONTROL
50µG/ML
48 HR
CONTROL
50µG/ML
96 HR
CONTROL
50µG/ML

observations of 3.3.3, indicating that stationary liquid cultures could be used to obtain a useful guide to biological activity.

4.4 Comparison of Falcarindiol, Falcarindiol diacetate and Griseofulvin

The antibiotic activity of these three compounds was compared using three different methods (see 3.3). The diacetate derivative appeared to be about ten fold less effective than falcarindiol in inhibiting spore germination in *M. gypseum* and *T. rubrum* whilst griseofulvin produced large inhibition zones at 5 µg/disc. (See Table 4.2).

Distinct differences were observed in the slide germination experiments. Falcarindiol completely inhibited the germination of the spores, with plasmolysis occurring at concentrations in excess of 25 µg/ml. The diacetate derivative did not totally inhibit spore germination even at 100 µg/ml but the germination percentage was reduced to less than 10%. However the spores exhibited pronounced swelling as if control over osmoregulation was disrupted. Spores treated with griseofulvin exhibited reduced and more variable germination rates, even at 100 µg/ml. In the griseofulvin-treated spores the subsequent germ tube growth was severely affected (see Plate 4.7) with pronounced curling of the growing tip of the germ tube. This phenomenom was observed at all concentrations from 5 µg/ml to 100 µg/ml.

Stationary liquid cultures exhibited a similar trend (Table 4.3). The griseofulvin treated cultures exhibited signs of spore germination but failed to develop the surface mycelial mat typical of untreated cultures. This observation was consistent with the slide germination results and the known properties of griseofulvin (89, 92).

Table 4.3

The Effect of Falcarindiol, its diacetate and Griseofulvin on Dermatophytes grown in liquid culture

	Falcarindiol			Diacetate			Griseofulvin			
	10	50	100	10	50	100	10	50	100	µg/ml
M. gypseum	+	-	-	++	+	-	-[1]	-[1]	-[1]	
T. rubrum	-	-	-	++	-	-	-[1]	-[1]	-[1]	

(- no growth, + moderate growth, ++ good growth of the fungus)
1 - visible indication of growth but no distinct mycelial formation

Table 4.2

The effect of Falcarindiol, its diacetate and Griseofulvin on Dermatophytes using the Assay Disc Method

M. gypseum

Falcarindiol µg/disc	Inhibition Zone Diameter mm	Falcarindiol-diacetate µg/disc	Inhibition Zone Diameter mm	Griseofulvin* µg/disc	Inhibition Zone Diameter mm
5	13	5	-	5	26
10	17	10	-	10	30
25	18	25	8	25	33
50	23	50	10	50	NT
100	25	100	13	100	NT

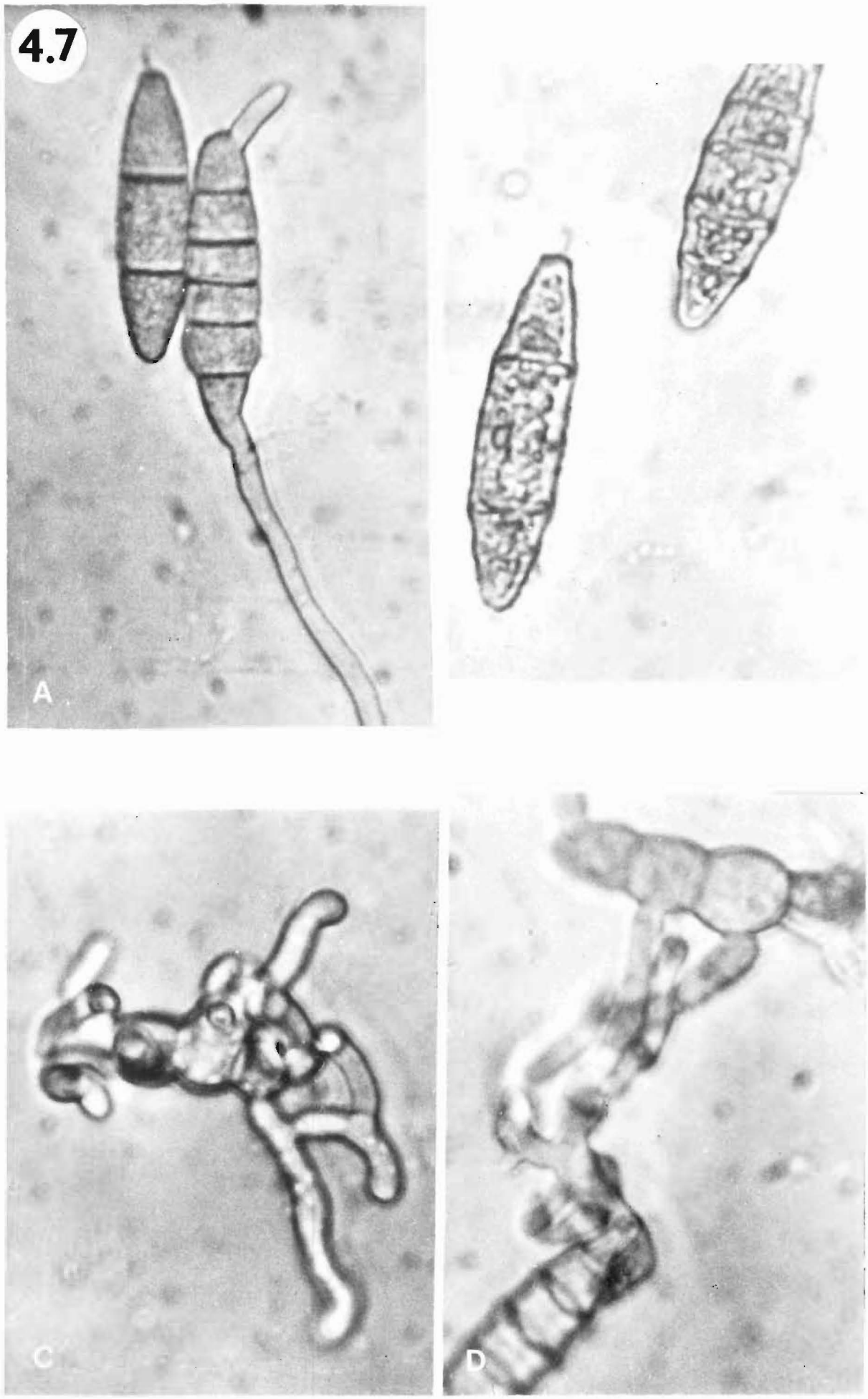
T. rubrum

5	17	5	-	5	28
10	22	10	-	10	31
25	24	25	-	25	33
50	30	50	19	50	NT
100	33	100	25	100	NT

NT = Not tested
results are the mean of three replicates
*NB: Griseofulvin causes the formation of a distinctive zone in which germination has occurred but subsequent germ tube growth inhibited.

Plate 4.7

Effect of Grisoefulvin and Falcarindiol on *M. gypseum*. A. Control (200x), B. Falcarindiol 40 µg/ml. (210X), C. Griseofulvin 50 µg/ul (170x), D. Griseofulvin 50 µg/ml (180x).


4.7
A
C
D

The TMS derivative (2.1.61) was not prepared in quantities sufficient to allow assessment of its biological activity. Because the TMS derivative did not prove to be a useful derivative for the present study, (3.4.3) investigation of its biological activity was not pursued further.

5.0 DISCUSSION

5.1 Folk Medicine

There has been a growing awareness in Western countries that so called 'primitive' people often possessed quite sophisticated folk medicine and made intelligent use of many of the medicinal plants within their area. As a consequence of this awareness, there has been growing interest in examining these medicinal plants for biologically active compounds (66, 164). The medicinal plants used in Nigeria (123), India (83, 98), Mexico (99), the West Indies (67, 75), and North America (111), have been the subject of a number of investigations, whilst the Chinese are known to be conducting extensive investigations of their herbal medicine. Unfortunately much of the latter work is not widely available due to language problems, however reports of investigations of *Panax ginseng*, the most famous chinese 'herbal' have been published in english (see 5.4.1).

The success of this investigation in isolating and identifying the active principle of *Schefflera digitata* as falcarindiol was due in part to the concept of this thesis having its origins in an examination of Maori folk lore medicine. Preliminary trials (41, 114), indicated that further investigation of the active principle was warranted. A search of the literature for information as to how the Maori's used *S. digitata* and what parts of the plant were used did not produce a very convincing picture however with conflicting reports as to its use.

5.2 Polyacetylenes

Polyacetylenes, with a few exceptions, have been found in very small amounts in most plants examined to date. It is most unusual for any one polyacetylene to be present in large amounts, and therefore the presence of falcarindiol at a concentration of 0.17% of the fresh weight of the leaves of *S. digitata* must make this plant almost unique. The only plant which is in any way comparable to *S. digitata* is the Umbellifer, *Falcaria vulgaris* (32) which contains large amounts of the ketone falcarionone (0.19% fresh weight) as well as a number of related C-17 polyacetylenes at concentrations of 5 to 600 ppm.

The lack of evidence for the presence of other polyacetylenes related to falcarindiol in extracts of *S. digitata* is also unusual, since many of the other plants investigated that contained C-17 polyacetylenes often contained several related compounds in approximately equal concentrations.

The primary aim of this thesis was to identify the biologically active principle, rather than to investigate the polyacetylenes of the New Zealand Araliaceae, and therefore the extracts have not been exhaustively fractionated to determine if any of the related polyacetylenes were present. Bentley *et al* (15) when fractionating *Daucus carota* extracts found that the related mono-alcohol, 3 acetoxy-8-ol and ketone derivatives of falcarindiol eluted from their columns before falcarindiol. The equivalent fractions from *S. digitata* extracts have been subjected to TLC, but have not yielded any compounds which gave the characteristic polyacetylene reaction with either isatin of phosphomolybdic acid. One is therefore left with the conclusion that if any of the related C-17 polyacetylenes are present they must be in very small quantities since it is possible to detect as little as 10 µg of falcarindiol by spraying with isatin. There may be another explanation for this apparent lack of related compounds; in this project all column chromatography was carried out with either 10% deactivated alumina or Florisil as absorbants, in preference to silica gel, since the latter was considered to be potentially capable of adverse catalytic activity, and therefore could possibly have resulted in rearrangement of the compound(s) originally present.

5.2.1 Chemotaxonomy of Polyacetylenes

The isolation of the polyacetylene falcarindiol from the Araliad *S. digitata* brings to nine the number of Araliaceae reported as containing polyacetylenes. Yosioka *et al* (128) examined extracts of a further nine species of Aralia by U.V. spectroscopy and found some evidence for acetylenes in several of them but the initial investigations have not, as yet, been followed up by the identification of the compounds responsible for the U.V. absorption. The polyacetylenes isolated are all C-17 compounds of the falcarinone type (30) (predominantly falcarinone itself) except for the C-18 compounds isolated from *Dendropanax trifidus* (103). The C-17 acetylenes are thought to be produced by decarboxylation of the corresponding C-18 polyacetylenic carboxylic acid (see Fig 1.1), so that the polyacetylenes isolated from *D. trifidus* do not represent a significant taxonomic difference. The isolation of falcarindiol from *S. digitata* is the first reported isolation of this compound from an Araliad, although it is not the first reported isolation of a diol from this group.

The failure to detect polyacetylenes from the other *Pseudopanax* species investigated (Table 3.4) needs to be confirmed by exhaustive fractionation of extracts of much larger samples before any taxonomic significance can be attached to this observation.

The isolation of falcarindiol from *S. digitata* conforms with the currently accepted taxonomic classification of this species (5) and does not alter the established concepts of the distribution of polyacetylenes. The high levels of falcarindiol found in *S. digitata* also indicates that other species of *Schefflera* should be investigated for polyacetylenes. An attempt was made to obtain a sample of a close relative of *S. digitata* from Queensland (Australia) but unfortunately the sample had been oven-dried and showed no activity when tested.

5.2.2. Isolation and Identification

The isolation and identification of acetylenic compounds from plant extracts is not as simple as is sometimes suggested. Highly conjugated polyacetylenes are often easily recognised by their distinctive U.V. absorption but this is not the case for interrupted chromaphores such as falcarindiol. In a search for biologically active compounds the lack of an intense U.V. absorption can be misleading and result in the possibility of compounds that are acetylenic in nature, being discounted. The other major problem is the general instability of the polyacetylenes, particularly their tendency to polymerise (10, 30). This can also provide a serious obstacle to the identification of biologically active polyacetylenes. It was fortunate, in this case that the active compound was present in unusually large amounts in *S. digitata*. The presence of many polyacetylenes in only trace amounts probably explains why biological activity has only been described for a small number of polyacetylenes. In many of the reported isolations often less than 20mg of material was obtained from quite large quantities of plant material. Thus although the chemotherapeutic or pharmacological activity of a plant may be known, it would be difficult to obtain sufficient of the compound for identification.

The classical techniques for identification of polyacetylenes involve the use of U.V. and 'H-NMR' spectroscopy, coupled with mass spectroscopy. With these techniques, it is possible for an established laboratory, experienced in identifying polyacetylenes, and with a large library of authentic compounds and spectra, to readily

establish the identity of very small quantities of an unknown polyacetylene, particularly if one has deliberately set out to look for polyacetylenes.

In the situation, such as presented in this thesis, where an unknown, biologically active compound was obtained, the easiest information to obtain; the Electron Impact Mass Spectrum and the U.V. spectrum, are of little value in identifying the compound, unless a range of authentic compounds are available for comparison.

A new mass spectroscopic technique which will soon become more widely available, that of High Resolution Chemical Ionisation Mass Spectroscopy (H.R.C.I.M.S.), can readily provide information such as the molecular weight and the molecular formula of the compound under investigation. Recent new developments in Negative Ion Mass Spectroscopy and Multiple Ion Monitoring offer new and exciting possibilities, which should make the identification of small quantities of biologically active compounds much easier.

Another new resonance technique which is revolutionising the identification of biologically active compounds present in slightly larger quantities is ^{13}C-NMR. When ^{13}C-NMR first became available in the early 1970's, large samples were required and the machines were few and expensive. Now however, the technique is much more widely available and the development of micro-probes and data handling systems means that samples of a few mg can now be analysed successfully. The advantage that ^{13}C-NMR offers is that not only does it allow assignment of all carbons in different chemical environments, but also by using SFORD conditions, the number of protons attached to each carbon can be determined.

The ^{13}C-NMR chemical shifts for a large number of compounds have now been determined, but the acetylenes have not yet been adequately investigated. Huntsman (93) recently reviewed the status of ^{13}C-NMR data on polyacetylenes. With the exception of the work of Hearn (96) the polyacetylenes investigated have been linear symmetrical polyacetylenes, in which the resonances of the acetylenic carbons amalgamate in pairs (29, 64, 189).

Hearn (96) investigated a number of symmetrical and asymmetric aromatic mono and di-acetylenes and observed that asymmetric

acetylenic carbons have characteristic individual resonances. This observation was confirmed by Zeisberg and Bohlmann (189), who investigated some simple asymmetric polyacetylenes and Miller *et al* (124), who examined the ^{13}C-NMR spectrum of a series of oxygenated acetylenic fatty acids of Isano oil.

The assignments made by Zeisberg and Bohlmann (189) are not however, in agreement with those made by other workers.

The assignment of the acetylenic carbon resonances of falcarindiol (3.4.3) was made by reference to the work of Hear (86) and Miller *et al* (124). There is also a lack of information and the effect on chemical shifts of hydroxyl groups β to double bonds contained within linear hydrocarbons.

Much of the work on the identification of polyacetylenes (30) was completed before the development of ^{13}C-NMR, which probably explains the lack of an adequate data base and, until the recent development of micro-probes, the requirement to have at least 20mg of sample for ^{13}C-NMR probably precluded its use as an identification tool.

Recent developments in ^{13}C-NMR and H.R.C.I.M.S. mean that in the near future, the preferred methods for identification of polyacetylenes will likely involve both these techniques.

5.3 Biological Action

Antibiotic activity has been attributed to a number of polyacetylenes (6, 7, 95, 96, 103, 104, 145, 166, 170) and in many cases this antibiotic action has been known for some time, as has the toxic nature of polyacetylenes (49, 53, 58). It would appear that the potential for some of these compounds as drugs was recognised (152), but the human toxicity of a few of these compounds seems to have discouraged further investigation of these compounds in a pharmaceutical and chemotherapeutic context. An attempt was made to determine the requirements for antibiotic activity (145), and some parameters were defined but the study concentrated on aromatic derivatives and did not adequately explore the linear polyacetylenes.

The mammalian toxicity of falcarindiol has not been determined but it would seem likely that it would be toxic. The toxin 'Caratatoxin' isolated from carrots by Crosby and Aharonson (58) was identified as the closely related C-17 monol falcarinol by Bentley *et al* (15).

Any toxic properties possessed by falcarindiol may not be important if the compound were used for topical applications but would preclude it from being used to control systemic fungal infections.

Polyacetylenes have been observed to possess a wide range of biological activities. Polyacetylenes have been shown to exhibit nematicidal activity (108, 109), while a common pentayne has ovicidal activity against certain insects (129). The mammalian toxicity of water hemlock (*Cicuta virosa* L) is due to the polyacetylene 'cicutoxin' (8, 53), while one of the fish poisons used in the lower Amazon region is an acetylenic pyran alcohol (49).

Polyacetylenes have also been found with phytoalexin type activity (6, 74), other polyacetylenes including falcarindiol exhibit antifungal activity against phytopathogens (103, 104), while others appear to stimulate spore germination of a number of fungi (22). The polyacetylenes include a wider range of chemical structures ranging from the hydrocarbon pentaynene types to alcohols, ketones and carboxylic acids. There are also lactones, thiophenes, aromatic derivatives and furanoacetylenes. The one feature that all the acetylenes containing ring structures have in common is that they all appear to be derived from linear polyacetylenes (30). This observation may explain some of the biological activity of compounds such as falcarindiol. For example; polyacetylenic glycols may cyclize to form dihydrofurans or furans in the presence of base, whilst amino groups can form cyclic products by addition to conjugated triple bonds. Thus it is possible that in a biological context the reactivity of the acetylenic groups and their ability to facilitate the formation of cyclic complexes is responsible for their biological activity.

The isolation of (137) and identification (138) of a D-glucose derivative of a linear polyacetylene from *Bidens frondosa* raises an interesting possibility as to how the plant may store polyacetylenic compounds. This, the second isolation of an acetylenic glycoside from a plant also raises a question mark regarding the adequacy of much of the polyacetylenic screening work since the presence of a sugar moiety would be expected to alter the solubility of the polyacetylene and hence its extractability in hydrocarbon solvents.

5.4 Araliaceae

5.4.1 Chemotaxonomy

The chemotaxonomic relationships of plant species is a subject of increasing interest among chemists and botanists, as new techniques make it possible to routinely identify a wide variety of compounds present in plants even though they may be present in very small amounts.

The chemotaxonomic relationships of the Araliad's have not been extensively investigated when compared to the amount of work done on the Umbelliferae (28, 59) and the Compositae (50).

A number of species of Araliaceae have been investigated and several compounds isolated. *Panax ginseng* has been the subject of several investigations with a number of ginsenosides (saponin glycosides) isolated from this and related species (167). The fatty acid composition has also been investigated (55), whilst several sapogenin glycosides have recently been isolated from *Tetrapanax papyriferum* (7a). Several other South East Asian Araliads have also been investigated with the isolation of aliphatic hydrocarbons, alcohols, fatty acids (182, 187), sterols, taraxerol, fatty alcohols (50) and a novel coumaran-glycoside 'innovanoside' (186) having been reported to date. The New Zealand Araliaceae have been investigated by Cambie and his co-workers (47) and a number of fatty acids, sterols, terpenes, waxes and aliphatic compounds isolated from six species of *Pseudopanax*. The phenolic content of a number of New Zealand Araliads were also investigated by Ng and Walker (131). The polyacetylenes found in the Araliaceae have already been discussed (see 5.2.1).

5.4.2 Biological Activity in Araliaceae

A number of different species have also been examined for biological activity, with both chemotherapeutic and pharmacological activity having been detected in crude extracts of several Araliaceae (23, 69, 121, 122, 136, 174). The initial detection of biological activity in these plants does not appear to have been followed up by isolation and identification of the active compound. The only other reported occurrence of antibiotic activity in an Araliad which has been investigated in detail is the isolation of two antifungal acetylenes from *Dendropanax trifidus* by Kawazu *et al* (103).

5.5 Antibiotic Activity of Falcarindiol

Falcarindiol was isolated and identified by virtue of its antifungal activity. The initial observations of the selective anti-dermatophytic activity of crude preparations were confirmed by subsequent tests with the purified compound. The dermatophytic fungi were found to be more sensitive to the antibiotic action of falcarindiol than were other fungal species.

Kemp (103) isolated two C-17 acetylenic alcohols, falcarinol and falcarindiol and tested them for activity against a range of phytopathogenic fungi. He found the diol to be a more potent inhibitor of fungal germination than the mono-alcohol; falcarinol. Crosby and Aharonson (58) and others have found falcarinol to be the toxic component of carrots, therefore either falcarindiol is even more toxic than falcarinol, or the animal toxicity and antifungal activity result from different properties of these molecules.

Direct comparison of the minimum inhibitory concentrations for falcarindiol against phytopathogenic fungi reported by Kemp (103) and the results of this thesis is not possible because of the different assay systems used, but some comparisons can be made. Kemp found that some fungi were completely inhibited by 10ppm of falcarindiol, while others, such as *Aspergillus niger* and *Botrytis cinerea* required higher concentrations before complete inhibition of germination occurred. It was not possible to test the fungi Kemp found to be most susceptible, but related species of *Cladosporium* and *Colletotrichum* were available and were tested for susceptibility to falcarindiol (Table 3.6 and 3.7). Both species were found to tolerate quite high concentrations of falcarindiol (100ppm).

Aspergillus niger was included as a test organism in both screening tests. In this study only inhibition of sporulation of the fungus grown on agar plates was observed at a concentration of 640ppm. In liquid culture no inhibition was detected at a falcarindiol concentration of 100ppm, whilst in the spore germination assay a variable effect was observed. It was also noticed that in the slide germination assay very low germination rates were obtained in the controls, suggesting that all the requirements for spore germination had not been met.

The apparent difference in concentrations which inhibit various species may reflect the differences in the Bioassay systems or it may have resulted from differences in the methods used to measure the concentration of, and handle falcarindiol. In this study the concentrations given were the theoretical concentrations obtained by diluting a large sample the concentration of which was obtained by weighting. It is assumed that significant losses due to polymerisation may have occurred and therefore the actual concentration of falcarindiol present may be as much as 20% less than the stated concentration. For this reason no errors are given and in many instances only the presence or absence of antifungal activity was established. Because of possible variations in the actual concentration of falcarindiol *M. gypseum* was included as a test organism in every bioassay set up as an internal reference so that valid comparisons could be made between the different experiments.

Another explanation of the differences in antifungal activity as determined by Kemp (103) and that observed in this study may be the compound itself. There are two chiral centres in the molecule, at the carbons bearing the alcohols, which means that there are four possible optical isomers. Since the optical rotation of the falcarindiol obtained in either study was not determined, it is possible that more than one optical isomer exists in nature and this may explain some of the observed differences in antifungal activity.

In human fungal infections, mycelium rather than spores, would be the target of any antibiotic so a bioassay was devised to test the effect of falcarindiol on the mycelial phase. Lack of a large amount of purified falcarindiol prevent extensive replicated experimentation, but the experiment devised (4.3) demonstrated that falcarindiol was capable of inhibiting mycelial growth.

TEM examination of the treated spores (4.2) suggests that falcarindiol has its primary effect on either the spore wall or the cytoplasmic membrane. Falcarindiol is a lipophilic compound and would be expected to associate strongly with the lipids of the membrane, thus it is likely that the primary effect is on the membrane structure. Polyacetylenes such as falcarindiol, have the potential to form cyclic compounds by addition to a variety of compounds (5.3) and such reactions could lead to the disorganisation of the spores membrane systems. If this occurs it could partly explain the observed antifungal activity.

The marked sensitivity of the dermatophytic fungi to falcarindiol must be the result of specific structural or functional

features which are characteristic of the dermatophytes and absent in other fungi. Although the dermatophytic fungi have long been recognised as a specific and unique group of organisms, studies to date, (1.5.12) have failed to find any differences in the composition of their cell walls and membranes, from other fungi, which could explain their unique sensitivity to falcarindiol.

Falcarindiol was also found to have bacteriostatic activity against *B. subtilis* and *S. aureus*, but when tested against Gram -ve bacteria, falcarindiol failed to inhibit their growth (Table 3.5). The nature of the antibacterial action was not investigated further.

The observed inability of falcarindiol to inhibit the growth of the yeast *Candida albicans* (Table 3.5) was in agreement with the observations of Tanaka *et al* (166) who found that a range of acetylenes failed to inhibit the growth of this organism.

5.6 Requirements for Antibiotic Activity in Polyacetylenes

Reisch *et al* (145) and Tanaka *et al* (166) tested over 110 synthetic acetylenes for activity against a range of micro-organisms including dermatophytic fungi and found that many of them possessed antibiotic activity. Highly unsaturated hydrocarbon acetylenes and aromatic derivatives were found to inhibit some of the dermatophytic fungi at concentrations of 5ppm, while simple terminal mono-acetylenes exhibited no activity. Symmetrical acetylenes were also found to have no detectable antibiotic activity. Tanaka *et al* (166) observed that maximum activity was obtained for di-acetylenes with secondary carbonyl functions α to the triple bond and/or with secondary alcohol functions between triple and double bonds. Reisch *et al* (145) suggested that the antifungal activity of acetylenes was enhanced by lipid solubility and was also improved by polarization of the triple bonds. These findings are consistent with the observation of this study, that acetylation of falcarindiol reduced the antifungal activity, whilst the structure of falcarindiol meets the requirements set down by Tanaka *et al* (166) for antifungal activity.

6.0 Conclusion

Falcarindiol was isolated from *S. digitata* as a result of an investigation of the antifungal activity of this plant species. The observed selective antifungal activity of falcarindiol suggests that polyacetylenes should be the subject of more detailed tests of their antibiotic activity, and that the mammalian toxicity of a greater range of polyacetylenes should be determined.

It is recognised that falcarindiol itself may not be suitable for further development as a commercial antifungal agent. The mode of action of falcarindiol appears, however, to be distinctly different from that of griseofulvin and other antifungal agents, so it is hoped that the discovery of this selective antifungal activity will encourage further investigations to determine the precise chemical requirements for antifungal activity. This could best be achieved by the synthesis of a range of analogs and derivatives of falcarindiol and testing them for antifungal activity. The often ignored glycosylated polyacetylenes also offer other possibilities for antifungal active derivatives.

In this project, testing of the antifungal activity of falcarindiol was confined to *in vitro* testing. It is hoped that in the near future tests may begin on laboratory animals so that the clinical significance of the observed antifungal activity may be determined. This will be necessary before any commercial development is contemplated, because many of the compounds observed to have *in vitro* antifungal activity have subsequently proved to be ineffective *in vivo*.

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APPENDIX A

Additional Reports of Isolation of Polyacetylenes from Species of the Family Compositae

A. Polyacetylenes from New Species

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Appendix B

Fungal Polyacetylenes

Since the publication of 'Naturally Occuring Acetylenes' by Bohlmann *et al* (30) the following reports on the isolation of polyacetylenes from fungal species have been published.

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Higham, C.A., Jones, E.R.H., Keeping, J.W., and Thaller, V. (1974) ibid 1991-1994.

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Appendix C

Mass Spectroscopy

Ions observed in the High Resolution Chemical Ionisation Mass Spectrum of (9Z)-3,8-diacetoxy-heptadeca-1,9-diene-4,6-diyne. (Falcarindiol diacetate) obtained on an AEI MS902 (see 3.4.31).

Observed Mass	Ion	Composition	Required Mass
362.2380	$M+NH_4^+$	$C_{21}H_{32}O_4N$	362.2331
345.2081	M+H	$C_{21}H_{29}O_4$	345.2066
344.1994	(M-H)-H	$C_{21}H_{28}O_4$	344.1987
302.2037	$M+NH_4$-acetic acid	$C_{19}H_{28}O_2N$	302.2120
301.1823		$C_{19}H_{25}O_3$	301.1804
285.1857	M-H-acetic acid	$C_{19}H_{25}O_2$	285.1854
284.1786	M-acetic acid	$C_{19}H_{24}O_2$	284.1776
283.1666		$C_{19}H_{23}O_2$	283.1698
279.1618		$C_{16}H_{23}O_4$	279.1596
261.1780		$C_{17}H_{25}O_2$	261.1854
261.1453		$C_{16}H_{21}O_3$	261.1491
260.1831		$C_{17}H_{24}O_2$	260.1776
259.1663		$C_{17}H_{23}O_2$	259.1698
243.1799		$C_{17}H_{23}O$	243.1749
242.1704		$C_{17}H_{22}O$	242.1671

High Resolution Mass Spectrum of (9Z)-3,8-diacetoxy heptadeca 1,9-diene-4,6-diyne, obtained by GCMS on an appropriately equipped MS32 mass spectrometer (see 3.4.31). The GLC separation was achieved on a 10 inch glass column of SE30 held isothermally at 220°C.

Observed Mass	Intensity	Composition	Required Mass
301.1821	86	C19 H24 03	301.1797
286.1926	68	C19 H26 02	286.1931
285.1855	178	C19 H24 02	285.1853
284.1770	241	C19 H24 02	284.1775
261.1858	64	C17 H25 02	261.1848
260.1731	292	C17 H24 02	260.1770
259.1738	537	C17 H23 02	259.1692
243.1746	131	C17 H23 01	243.1743
242.1636	342	C17 H22 01	242.1665
241.1634	247	C17 H21 01	241.1587
234.1594	80	C15 H22 02	234.1612
233.1578	84	C15 H21 02	233.1534
226.1733	468	C17 H22	226.1716
225.1612	172	C17 H21	225.1638
224.1569	114	C17 H20	224.1560
213.1095	130	C11 H17 04	213.1122
203.0705	179	C12 H11 03	203.0705
201.1041	61	C13 H13 02	201.0912
200.0793	69	C13 H12 02	200.0834
199.0810	271	C13 H11 02	199.0756
185.0853	187	C 9 H13 04	185.0810
181.1009	122	C14 H13	181.1014
179,1443	152	C12 H19 01	179.1431
178.1338	128	C12 H18 01	178.1352
175.0758	702	C11 H11 02	175.0756
172.0187	302		
171.0761	634	C12 H11 01	171.0807
168.0803	144	C 9 H12 03	168.0783
167.0784	334		
165.0679	284	C13 H 9	165.0702
162.0693	364	C10 H10 02	162.0678
161.1294	66	C12 H17	161.1326
161.0620	891	C10 H 9 02	161.0600
158.0745	804	C11 H10 01	158.0729
157.0693	1601	C11 H 0 01	157.0651
155.0878	445	C12 H11	155.0858
153.0718	770	C12 H 9	153.0702
152.0641	554	C12 H 8	152.0624
147.0810	280	C10 H11 01	147.0807
145.0649	383	C10 H 9 01	145.0651
144.0530	296	C10 H 8 01	144.0573
143.0714	404	C 7 H11 03	143.0705
142.0739	695	C11 H10	142.0780
141.0659	1650	C11 H 9	141.0702
139.0504	507	C11 H 7	139.0546
129.0613	1137	C10 H 9	129.0702
128.0541	1714	C10 H 8	128.0624
127.0467	753	C10 H 7	127.0546
117.0686	544	C 9 H 9	117.0702
116.0262	515	C 9 H 8	116.0624
115.0523	2393	C 9 H 7	115.0546
102.0463	608	C 8 H 6	102.0468

High resolution mass spectrum of the TMS derivative of Falcarindiol obtained by GCMS on an MS32 machine. GLC separation was achieved on a 5% SE-30 column with a temperature programme beginning at 180°C and rising 4°C/minute.

Observed Mass	Intensity	Composition	Error
258.1694	131	C13 H26 03 Sil	4.3
244.1804	106	C17 H24 01	-2.3
243.1738	196	C17 H23 01	-1.1
242.1694	642	C17 H22 01	2.3
241.1579	136	C17 H21 01	-1.3
240.1586	212	C13 H24 02 Sil	4.1
232.1799	100	C16 H24 01	-2.8
226.1699	297	C17 H22	-2.2
225.1663	106	C17 H21	2.0
224.1573	154	C17 H20	0.8
213.1551	186	C12 H21 03	2.0
203.1307	104	C10 H19 04	2.4
199.1116	198	C10 H19 02 Sil	-3.8
186.1421	238	C14 H18	1.2
		C10 H22 01 Sil	-1.9
185.1024	285	C 9 H17 02 Sil	2.6
175.0777	384	C11 H11 02	1.8
173.0880	364		
172.0851	354	C12 H12 01	-3.7
171.0831	2007	C12 H11 01	2.1
167.0843	612	C13 H11	-1.8
165.0705	428	C13 H 9	0.1
161.0652	1093	C10 H 9 02	5.0
		C 6 H13 03 Sil	1.8
159.0724	553		
158.0795	989	C 7 H14 02 Sil	3.2
157.0703	3050	C11 H 9 01	4.9
155.0835	891	C12 H11	-2.6
154.0739	402	C12 H10	-4.4
153.0702	855	C12 H 9	-0.2
152.0584	708	C12 H 8	-4.2
147.0744	701		
145.0699	834	C10 H 9 01	4.6
143.0772	1927		
142.0733	1362	C11 H10	-4.9
141.0650	2680		
139.0491	608		
133.0479	1542	C 8 H 9 Sil	0.6
130.0481	1466	C 5 H10 02 Sil	3.1
129.0376	4095*	C 5 H 9 02 Sil	0.4
128.0280	4095*	C 9 H 4 01	1.8
127.0363	2321	C 6 H 7 03	-3.2
		C 5 H 8 01 Sil	-1.4

* Accuracy of these masses suspect (Resulting from possible saturation of the detector).